DNA RECOVERY FROM LATENT BLOOD AFTER IDENTIFICATION BY FLUORESCEIN

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DNA RECOVERY FROM LATENT BLOOD AFTER IDENTIFICATION

BY FLUORESCEIN

A

THESIS

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Abstract

Luminol has been widely used in the field of crime scene investigations to detect latent blood; however, luminol has the tendency to destroy DNA evidence. Fluorescein, an alternative to luminol for detecting latent blood at a crime scene, had not been adequately evaluated for its impact on DNA evidence. This thesis demonstrates the successful recovery of DNA from a blood sample treated with fluorescein. DNA was extracted from blood-containing denim substrates after fluorescein was applied to the substrates. The DNA locus, D18S51, was amplified using standard polymerase chain reaction (PCR) techniques, separated by gel electrophoresis, and visualized using ultraviolet light. The results demonstrate that DNA was successfully recovered from the samples.

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List of Abbreviations

A	Adenine
ALS	Alternative Light Source
bp	base pair
C	Cytosine
°C	Degrees Celsius
CODIS	Combined DNA Index System
DI	Deionized
DNA	Deoxyribose Nucleic Acid
dNTP	deoxyribonucleotide triphosphate
ddNTP	dideoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
G	Guanine
MSDS	Material Safety Data Sheet
P1	Primer 1 (Forward Primer)
P2	Primer 2 (Reverse Primer)
PCR	Polymerase Chain Reaction
RPM	Revolutions per Minute
STR	Short Tandem Repeats
T	Thymine
TAE	Tris-acetate EDTA

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In Loving Memory of

Dr. Douglas Schamel

1949 – 2005

Chapter 1

INTRODUCTION

Violent crimes often go unsolved because of the lack of evidence leading to a perpetrator. Therefore, any evidence found at the crime scene is vital to solving the crime. Violent crimes often leave latent (not visible to the naked eye) evidence, such as blood, at the crime scene. The identification and collection of the latent evidence is the responsibility of the crime scene investigator. New methods for obtaining evidence and identifying its source make the crime scene investigators job easier by providing more quantitative evidence. This thesis investigates the effect of a new method for identifying latent blood evidence on the ability to recover deoxyribonucleic acid (DNA) from the blood evidence.

Currently, luminol is used in the field of crime scene investigations to detect latent blood. When luminol is applied to blood, the blood will luminesce making the presence of blood obvious to the investigator. However, conflicting studies have shown that luminol, when applied to a latent blood sample, causes the DNA in the blood to be degraded. This causes the loss of DNA that can be used to identify a perpetrator. Budowle *et al.* (2000) reports that DNA can be recovered after substrates are treated with luminol. However, Schiro (2004) states that luminol can cause the loss of several genetic markers. Genetic markers are defined locations on chromosomes having known genetic characteristics [Rudin, 2002]. Most likely this is because repeated applications of the luminol is needed to maintain visualization. Each application of the luminol causes a chemical reaction between the luminol and the blood that could be destroying the sample's DNA. This is a major drawback to using luminol because any trace blood discovered through the use of luminol could be useless for DNA recovery, the ability to collect intact DNA for testing.

Unlike luminol, fluorescein, a new alternative method for latent blood detection [Cheeseman and Tomboc, 2001], has not been adequately tested for its impacts on DNA recovery. The goal of this study was to determine whether DNA could be recovered from latent blood samples that have been treated with fluorescein and a catalyst, hydrogen peroxide. The approach used in this study was to treat denim substrates with blood, use fluorescein and hydrogen peroxide on the denim to confirm the presence of blood, and extract the blood from the denim. After the blood was extracted, a polymerase chain reaction (PCR) was used to amplify a specific region of the DNA, locus D18S51 (see section 1.2), obtained from the blood. Then, gel electrophoresis was used to separate the DNA fragments and ultraviolet light was used to determine if DNA fragments were obtained from the blood sample. This analysis demonstrated that DNA could be recovered from the fluorescein-treated blood samples.

1.1. Blood

Blood is one of the primary types of biological, and latent, evidence found at crime scenes. Blood is the fluid consisting of plasma, blood cells, and platelets that is circulated by the heart through the vertebrate vascular system, carrying oxygen and nutrients to and waste materials away from all body tissues [The American Heritage®

Dictionary of the English Language, 2000]. Blood represents approximately 8% of a human's total body weight and has a volume of 5 to 6 liters in males and 4 to 5 liters in females. The normal pH of blood is 7.4. The function of blood in the human body is to: (1) distribute oxygen and nutrients to body tissues, remove metabolic wastes, and transport hormones, (2) regulate body temperature, pH, and volume, and (3) protect the body from infections and diseases. [Bevel and Gardner, 2001]

Blood is composed of plasma (55%) and formed elements (45%) (see Figure 1.1). Plasma is a straw-colored substance consisting of 91% water, 8% proteins, 1% salts, and 1% organic acids. Proteins in the plasma are made mostly by the liver and include albumin, globulins, and clotting proteins such as fibrinogen. [Bevel and Gardner, 2001]

Formed elements include three major components: (1) leukocytes (white blood cells), (2) platelets, and (3) erythrocytes (red blood cells). Leukocytes defend against disease and infections by engulfing and digesting bacteria and fungi [The American Heritage® Dictionary of the English Language, 2000; Marieb, 2003].

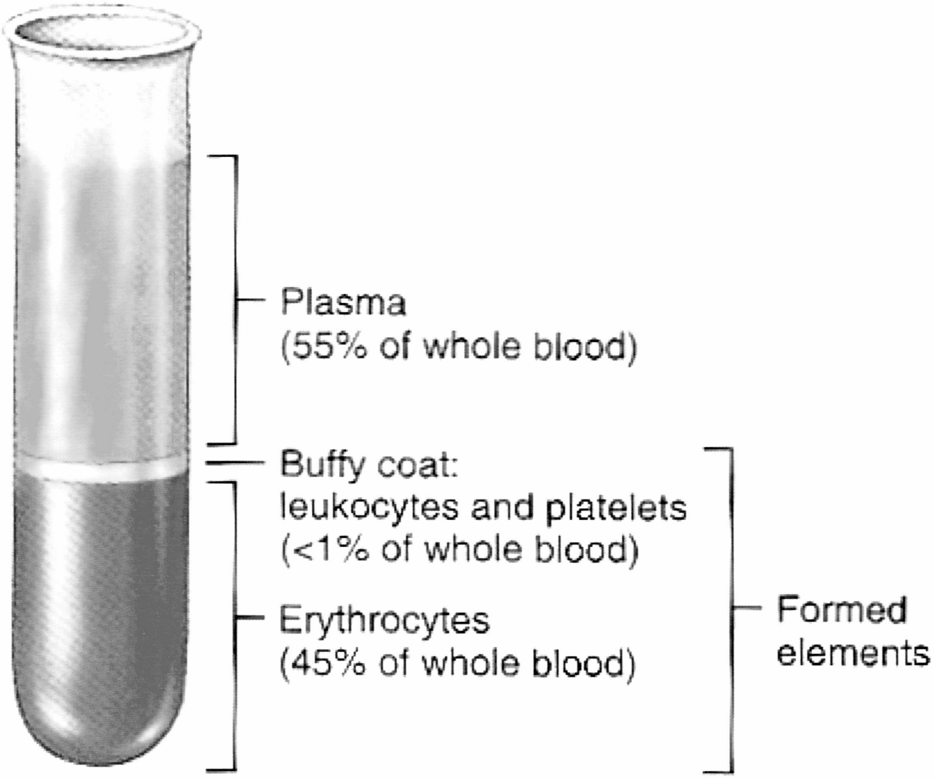

Figure 1.1. The components of blood are plasma, leukocytes, platelets, and erythrocytes. [from Marieb, 2003]

Platelets are fragments of megakaryocytes. A megakaryocyte is a large bone marrow cell that gives rise to blood platelets [The American Heritage® Dictionary of the English Language, 2000]. When a blood vessel is damaged, platelets form a "plug" to prevent blood loss [Wonder, 2001]. Hemostatis is the technical term for the body's attempt to prevent blood loss. There are three major phases of hemostatis: (1) muscular spasms, (2) platelet plug formation, and (3) blood coagulation. Muscle spasms accumulate platelets at the site of injury so they can bind together to form the plug. [Bevel and Gardner, 2001] The extent of hemostatis assists investigators in determining how old the blood is at crime scenes.

The major function of erythrocytes is oxygen transport. In the lungs oxygen binds to iron atoms in hemoglobin molecules. In body tissues, oxygen dissociates from iron providing oxygen to the tissues. A single red blood cell can have 250 million hemoglobin molecules [Marieb, 2003]. The structure of hemoglobin and a heme group are shown in Figure 1.2. A heme group is the deep-red, oxygen-carrying, non-protein, ferrous component of hemoglobin [The American Heritage® Dictionary of the English Language, 2000]. Each hemoglobin molecule can bind four oxygens, one per heme group [Marieb, 2003]. Understanding the effects of heme groups in blood is important to this study since fluorescein also binds to the iron in the heme group [James and Nordby, 2002].

Crime scene investigators are interested in blood found at crime scenes because blood contains valuable information about the origins of the blood: who it came from and how it got there. Blood has great evidential value when a transfer of blood between a victim and suspect can be demonstrated. DNA identifying the victim or suspect can be extracted from the blood collected at the crime scene. The red blood cells in a blood sample actually lack DNA; DNA is contained in the nucleus of white blood cells [Marieb, 2003].

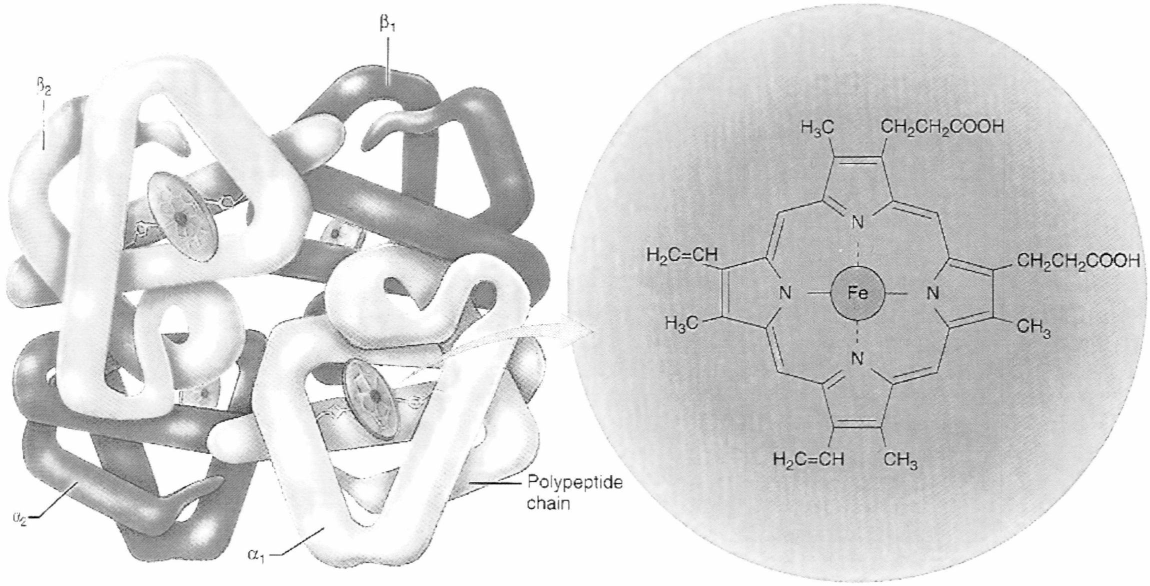

Figure 1.2. The structure of hemoglobin (left) consists of four globin chains. Each globin chain possesses an iron-containing heme group (right). [from Marieb, 2003]

1.2. DNA

Our criminal justice system is best described as a search for the truth. Increasingly, the forensic use of DNA is an important ally in that search [Connors *et al.*, 1996]. The development of DNA technology furthers the search for truth by giving investigators new tools for identifying victims and perpetrators of crimes. Through the use of DNA evidence, prosecutors are often able to conclusively establish the guilt of a defendant.

DNA is found in every cell of our body (with the exception of red blood cells, which lack nuclei) [Butler, 2002]. DNA is a nucleic acid that carries the genetic information in the cell and is capable of self-replication. Nucleic acids are long, single or double-stranded chains of different kinds of nucleotides joined one after the other at their phosphate groups. They differ in which nucleotide base follows next in sequence

[Weaver, 2002]. A nucleotide is composed of a sugar molecule, a phosphorous-containing group, and a nitrogen-containing molecule called a base [Saferstein, 2001; Weaver, 2002]. The sequence of nucleotides determines individual hereditary characteristics. The nucleotide sequence encodes the instructions for assembling proteins and, ultimately, new individuals of a species.

DNA consists of four bases: adenine (A), guanine (G), thymine (T), and cytosine (C) (see Figure 1.3). Adenine and guanine are purines, compounds with a 5-member ring connected to a 6-member ring base. Thymine and cytosine are pyrimidines, compounds with a 6-member ring base (see Figure 1.3). The nucleotides attach to each other in specified base pairs; A always binds to T and C always binds to G through hydrogen bonding. This pairing is called complementary. A complementary DNA strand is a strand that is matched to another DNA strand according to the base pairing rule. The base pairing between two nucleotide strands in DNA is constant for all species. However, the sequence of bases is different from species to species. [Weaver, 2002]

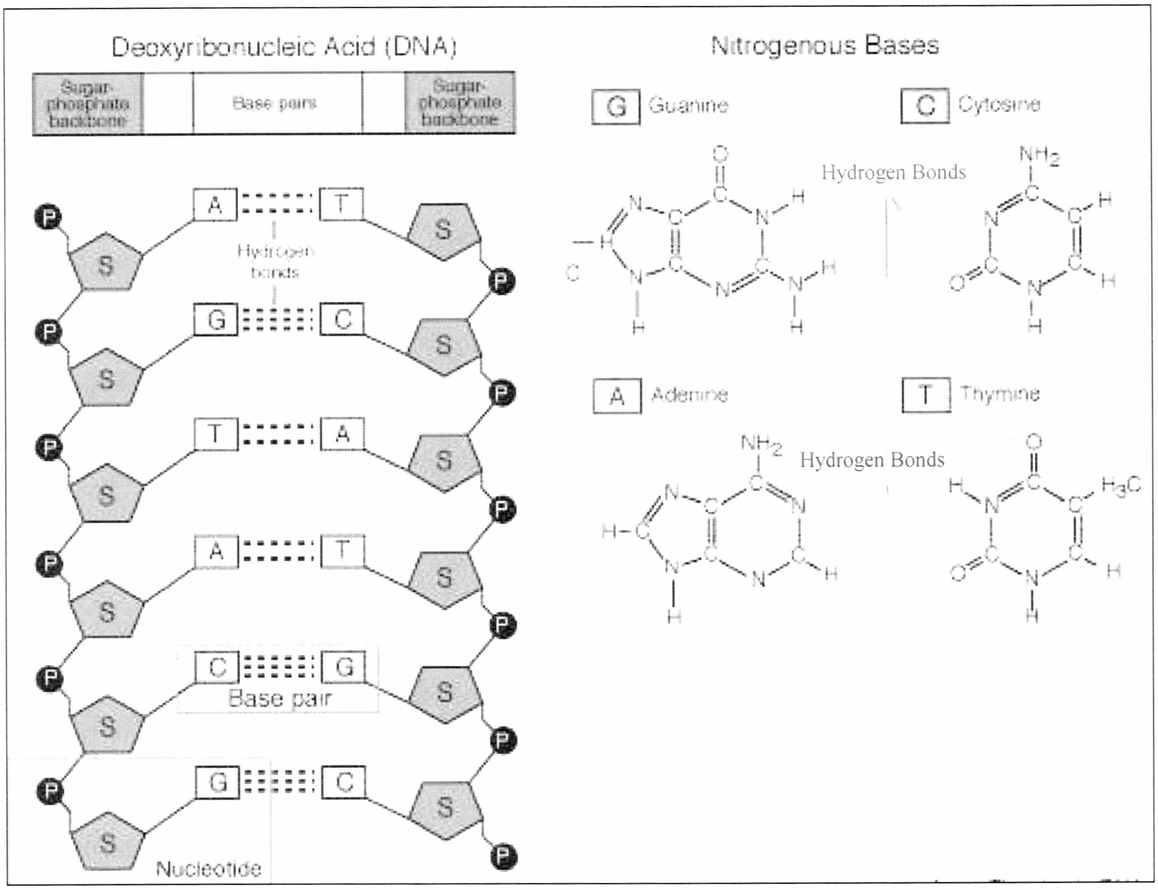

Figure 1.3. The DNA bases, adenine (A), guanine (G), thymine (T), and cytosine (C), are paired A-T and C-G by hydrogen bonding. [from Leja, 2003]

DNA is replicated with the help of enzymes and other proteins. An enzyme is a class of proteins that greatly speeds up reactions between specific substances, usually at their functional groups. A functional group is an atom or group of atoms that has a characteristic chemical reactivity [McMurry, 1999]. When DNA is replicated, each double-stranded DNA molecule begins unwinding and separating into two single nucleotide strands because of the enzyme called helicase [Garrett and Grisham, 1995]. A new, complementary strand of DNA is assembled by the enzymes DNA polymerase and ligase on the exposed bases of each parent strand according to the base-pairing rules

[Garrett and Grisham, 1995]. DNA is read from the 5' end to the 3' end. The 5' end is the hydroxyl on the phosphate group and the 3' end is the hydroxyl on the sugar group. So the new strands are assembled fro the 5' end to the 3' end by the enzymes. This leads to the formation of two identical DNA strands.

DNA is destroyed by nucleases. Nucleases are enzymes found in cells that degrade DNA, if magnesium is present, to allow for recycling of the nucleotide components. This is why it is common practice to store blood in a preservative called ethylenediaminetetraacetic acid (EDTA). EDTA chelates all of the free magnesium in a blood sample and prevents the nucleases from destroying the DNA in the sample. [Butler, 2001]

A chromosome is a threadlike, linear strand of DNA and associated proteins (shown in Figure 1.4) located in the nucleus of eukaryotic cells. The chromosomes carry the genes and function in the transmission of hereditary information.

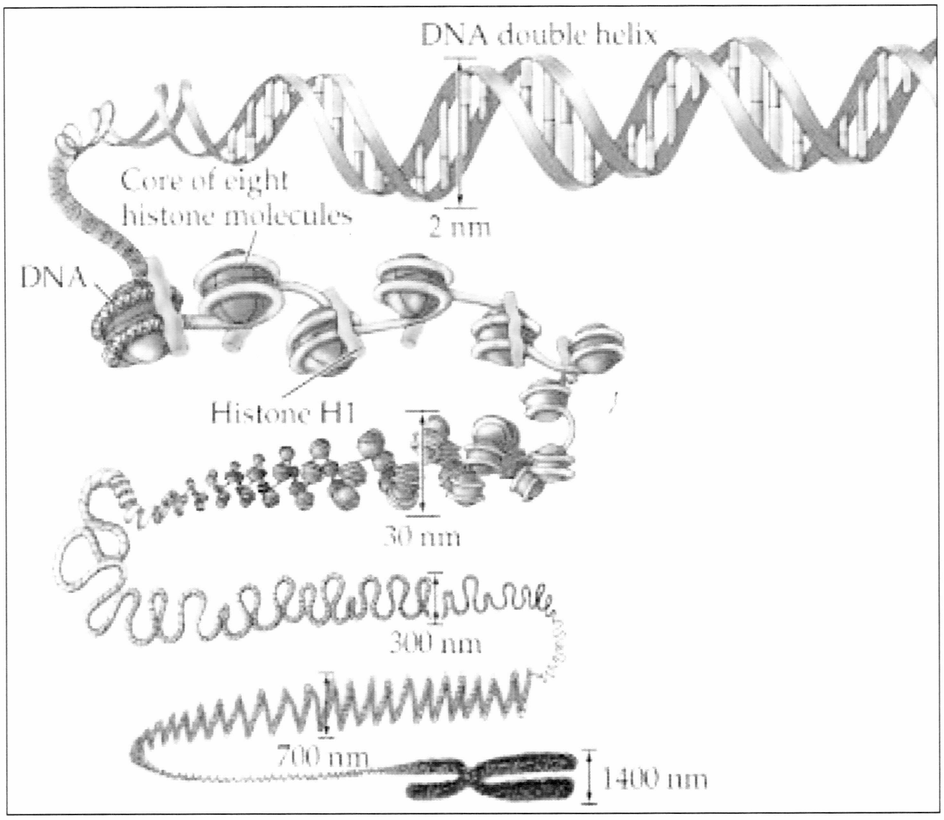

Figure 1.4. DNA is packed and coiled around histones. Histones, simple proteins containing basic amino acids, complex with the DNA and pack the DNA into tight masses of chromatin having the structure of coils, like a tangled telephone cord [The Columbia Electronic Encyclopedia, Sixth Edition Copyright © 2003]. This figure also shows the respective sizes involved. [from CIS Courseware Knowledge Systems Institute, 2005]

A normal person has 23 pairs of chromosomes (see Figure 1.5). Autosomes are chromosomes that are the same in both sexes (chromosome pairs 1 through 22). The 23^{rd} pair contains the sex chromosomes, the X and Y chromosomes. Males have an X-Y chromosome pair while females have an X-X chromosome pair. The Y chromosome is significantly smaller than X chromosome (see Figure 1.5). [Russell, 2002]

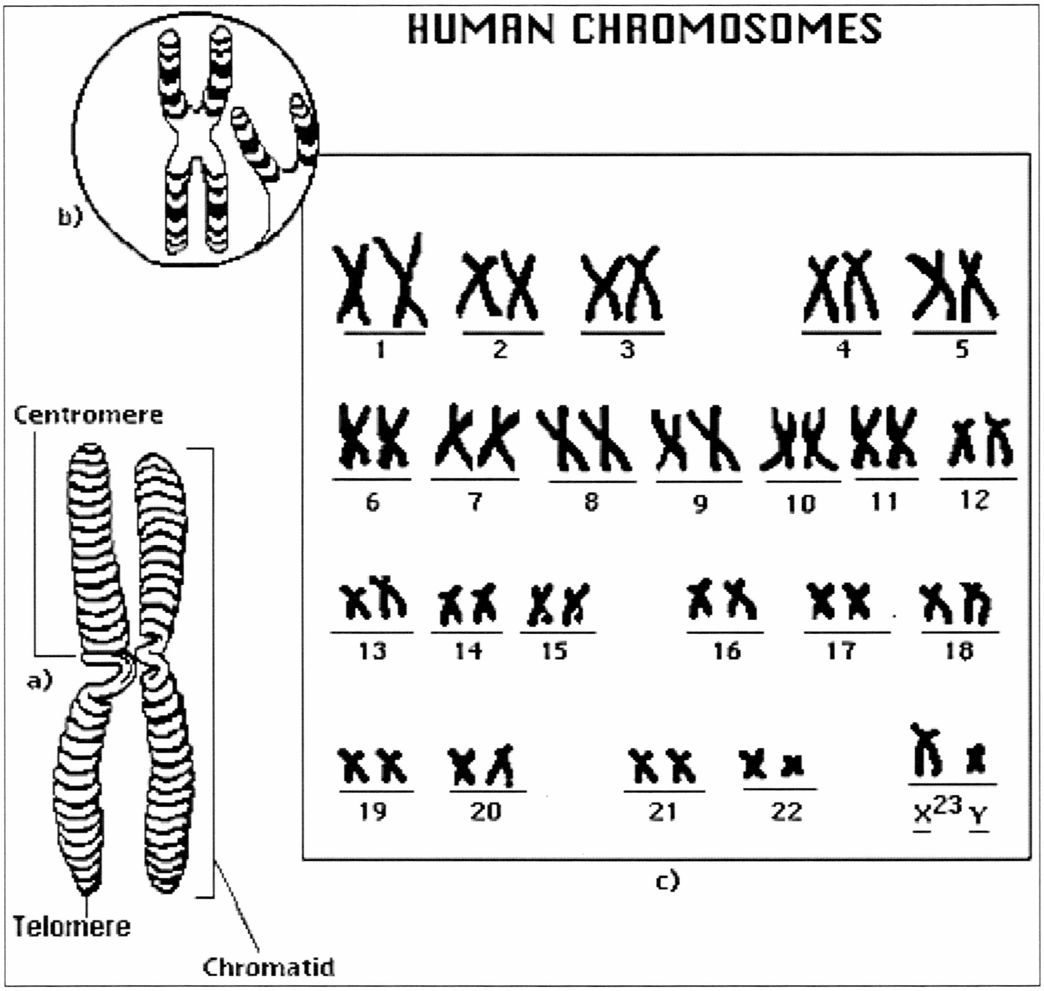

Figure 1.5. Diagram of the 23 chromosome pairs found every normal person. a) illustrates the location of a centromere (the center) and a telomere (the end) on each single chromosome (called a chromatid), b) shows an enlarged view a chromosome with various bands, and c) shows the number of chromosomes in humans and how they are paired. [from The National Health Museum, 2003]

Determining a suspect's gender can be done by conducting a chromosomal smear to look for the sex chromosomes. A chromosomal smear allows chromosomes to be viewed under a microscope when they are dividing. Only dividing chromosomes are visible because they condense during the prophase of meiosis. [Bloom, 2005]

Genes are units of information about specific traits that are passed down from parents to offspring. They are composed of specific DNA sequences and occur at specific locations (called loci) on a chromosome. An allele is one of two or more slightly different molecular forms of a gene that code for different versions of the same trait at a given locus on a chromosome. The allele frequency of a given locus is the relative abundance of each kind of allele carried by the individuals of a population. The frequency of a combination of alleles at different loci becomes increasingly small. For example, if the frequency of an allele at one locus is 1 in 1,000, and the frequency of the allele at a second locus is also 1 in 1,000, the frequency of the combination of alleles at both loci will be 1 in 1,000,000. With increasing numbers of loci, the frequencies of the combinations are correspondingly lower. [Adams, 2002]

The 13 Short Tandem Repeats (STR) loci on DNA strands employed by the Federal Bureau of Investigation (FBI) for DNA profiling are: CSF1PO, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, FGA, THO1, TPOX, and VWA [Budowle and Moretti, 1999]. Short tandem repeats consist of 2 to 5 base pair sequence that repeat a number of times depending on the individual.

DNA profiles consisting of records of individuals' STR characteristics are kept in the National DNA Databank, the Combined DNA Index System (CODIS). The loci comprising these profiles are also used for analyzing biospecimens found at crime scenes (see Figure 1.6) [Budowle and Moretti, 1999]. Employing the same 13 STR loci in all DNA analyses provides a standard method for typing of all felons, for identifying individuals, and identifying family members. Absolute certainty in DNA identification is not possible in practice; virtual certainty is possible due to the very small probabilities of a random match [Butler, 2001].

According to the literature, it is possible to recover DNA, "typeable at all 13 core CODIS STR loci" [Budowle, 2000]. This experiment focused on one of the 13 STR loci, D18S51. The D18S51 locus was chosen based on its use in other studies.

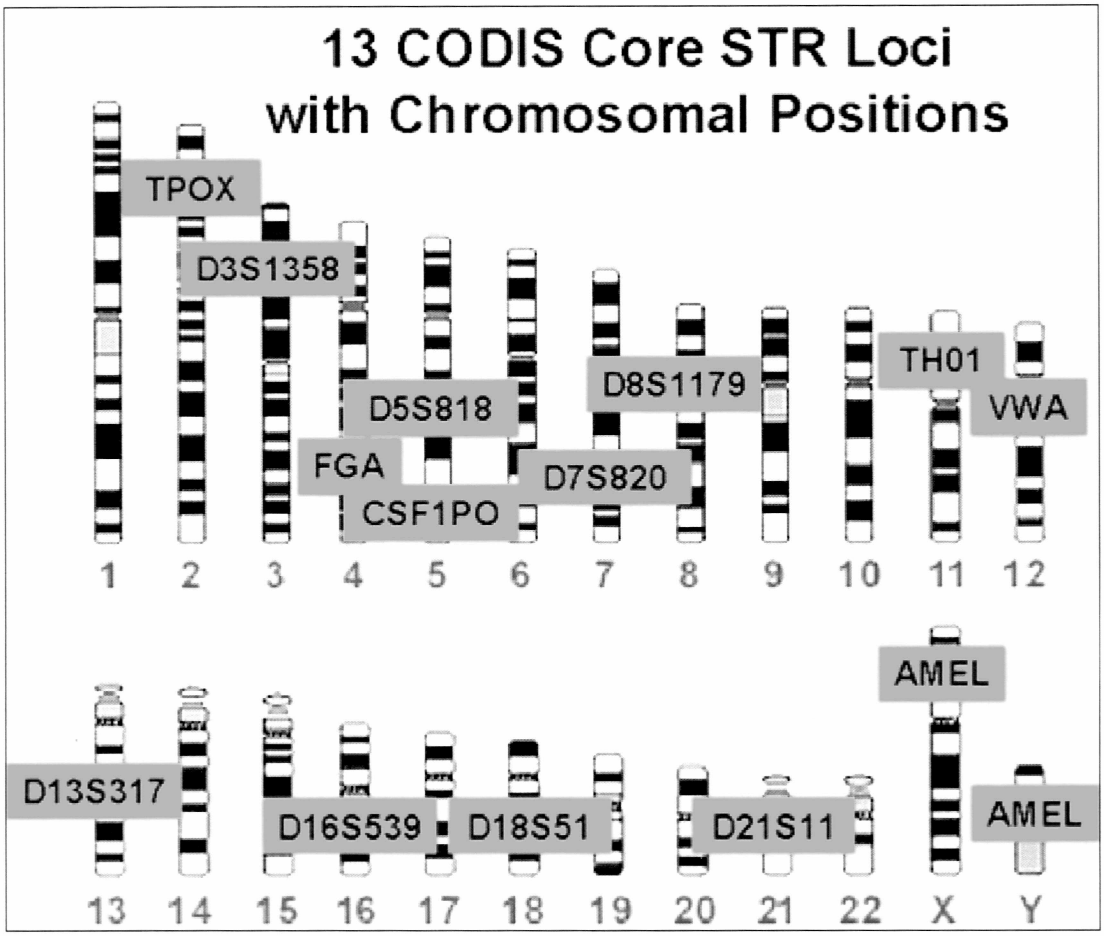

Figure 1.6. The chromosomal positions of the 13 CODIS Core STR Loci employed by the FBI for DNA analysis are shown on the 23 chromosome pairs (shown as one chromosome each except for the 23rd pair, X and Y). [from National Institute of Standards and Technology, 2005]

1.3 DNA Extraction and Amplification Techniques

Polymerase Chain Reaction (PCR) is the process of amplifying, making multiple copies, a very small amount of DNA into an amount great enough to analyze. The overall process involves mixing target DNA with an excess of primers, nucleotides and enzymes. The mixture is heated to separate the two strands of the DNA. The mixture is then cooled to allow the primers, short single strands of nucleotides expected in the target DNA, to bind to their complementary target sequences in the separated strands. Primers bind to specific nucleotide sequences in DNA. The enzymes manufacture a new strand of DNA starting from the point at which the primer has attached to the portion of DNA to be replicated. Nucleotides then added next to the primer by the enzymes and are used to build the complementary strand of DNA. [Coyne *et al.*, 2002] This process is repeated multiple times until the amount of DNA extracted from a sample has been increased to an amount that can be analyzed.

1.3.1 DNA Extraction

Prior to subjecting the sample to PCR, the DNA must be extracted from the sample. DNA molecules must be separated from proteins and other materials before examination because proteins that protect DNA in the environment of the cell can inhibit the ability to analyze DNA [Butler, 2002].

There are three primary DNA extraction methods: organic extraction, chelex extraction, and Flinders Technology Associates (FTA®) paper. A schematic of these extraction methods are shown in Figure 1.7. This study used the chelex extraction method. The chelex method was chosen because of the substrates used in this study. The blue dye in the denim substrates acts as a PCR inhibitor; however, the chelex method is able to remove this inhibitor while the other methods could not remove it [American Academy of Forensic Sciences, 1999].

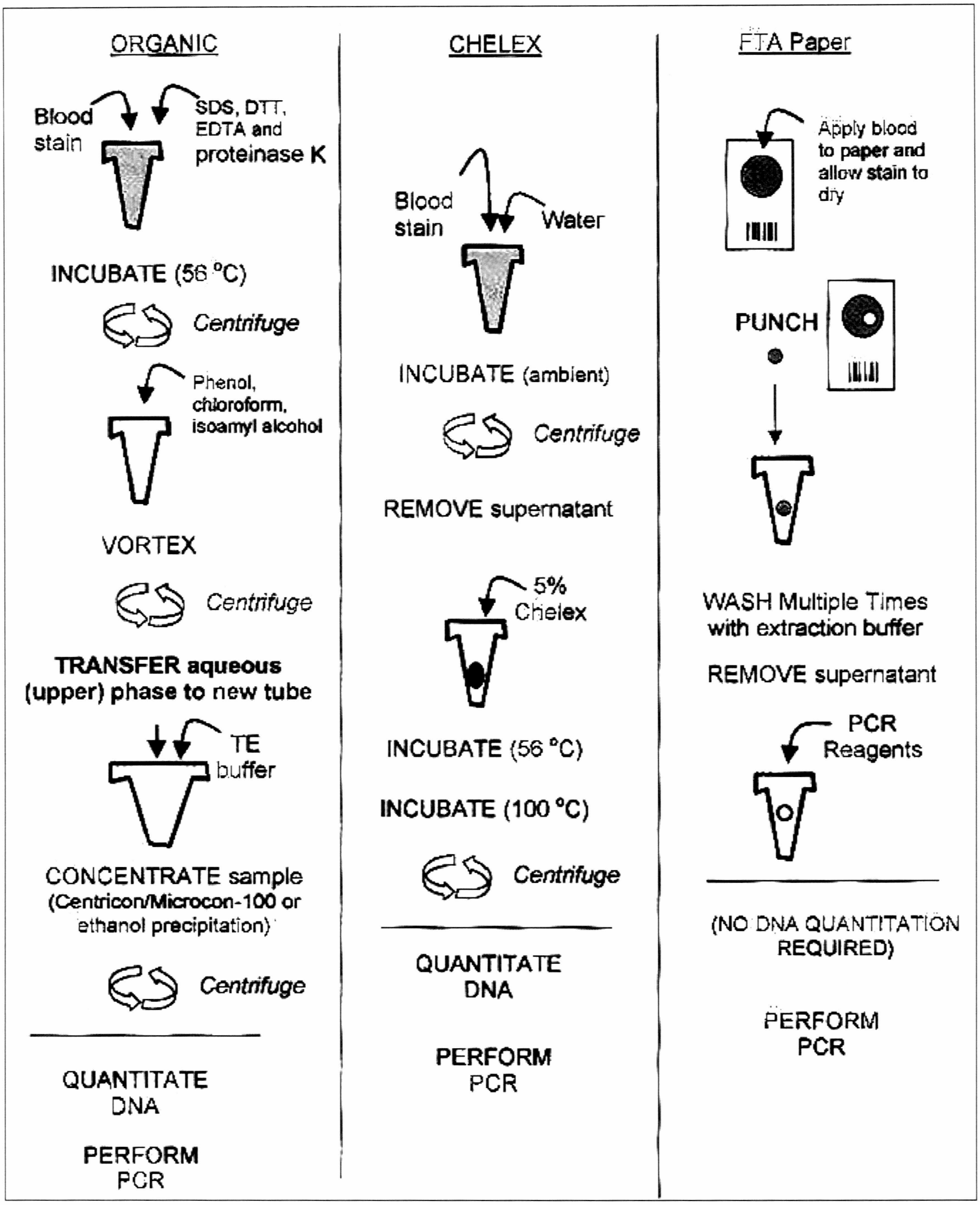

Figure 1.7. Common methods used to extract DNA include organic extraction, chelex extraction, and FTA paper. SDS stands for sodium dodecyl sulfate and DTT stands for dithiothreitol [from Butler, 2002].

Chelex is an ion-exchanging resin that binds metal ions such as magnesium. By removing magnesium from the reaction, DNA nucleases are inactivated so as to prevent DNA breakdown. Chelex extraction improves PCR because it removes PCR inhibitors. However, the chelex solution itself can inhibit the PCR process so it is important to remove the chelex solution from the extracted sample before it is subjected to PCR. [Butler, 2001]

1.3.2 PCR

After the DNA has been extracted from the sample, the DNA is combined with a mixture of components necessary for PCR. These components include: buffers, primers, dNTPs, and Taq polymerase.

The buffers, consisting of various salts and acids (such as Tris-HCl, $MgCl_2$, and KCl) are used to keep the DNA at a specific pH. [James and Nordby, 2002]. Magnesium ions stabilize DNA and are needed for optimal DNA polymerase activity [Russell, 2002]; however, high Mg^{2+} concentrations promote incorrect annealing of the primer to its site resulting in extraneous bands and low product yield. If too little Mg^{2+} is used, it decreases the tendency for the strands to stay together which results in lower yield. Typically an excess of Mg^{2+} is used to prevent any degraded DNA from consuming the magnesium and leaving the reaction mixture deficient in magnesium. [Saferstein, 2002] Because the magnesium in an article of evidence is removed during the chelex extraction process, the magnesium needed for PCR is added to the PCR mixture as a component of the buffer solution.

Primers are specific sequences of bases that bind to the template DNA once it has separated into two single strands. PCR requires two primers; forward and reverse. Since DNA is read from the 5' to the 3' end, the forward primer attaches to the 5' end of one of the two single DNA strands at the area of interest while the reverse primer attaches to the 5' end of the other DNA strand at the area of interest.

Deoxynucleotide triphosphates (dNTPs) are the nucleotides (adenine, cytosine, guanine, and thymine triphosphates) that are required to build the complementary strands of DNA [James and Nordby, 2002].

Taq polymerase is a thermo-stable enzyme derived from *Thermus aquaticus* and is produced as a recombinant product from *E. coli* over expressing the *Thermus aquaticus* (Taq) polymerase. The Taq polymerase begins adding nucleotides to the primer and eventually makes a complementary copy of the template. Taq polymerase withstands repeated incubations at 95°C without a significant decrease in enzyme activity and is suitable for routine PCR [Promega, 2001].

Once the all the components of the reaction mixture are ready, the samples are loaded into a thermocycler. A thermocycler is an instrument that repeatedly cycles through the various temperatures required for an iterative, temperature-dependant chemical process such as the polymerase chain reaction [Nanoword, 2005].

During the first step in PCR, denaturing, double-stranded DNA molecules are heated up to 94°C which separates or denatures them by breaking the hydrogen bonds between the base pairs so that they become single strands (see Figure 1.8). These single strands of DNA then become the templates for the formation of the new DNA strands [Russell, 2002].

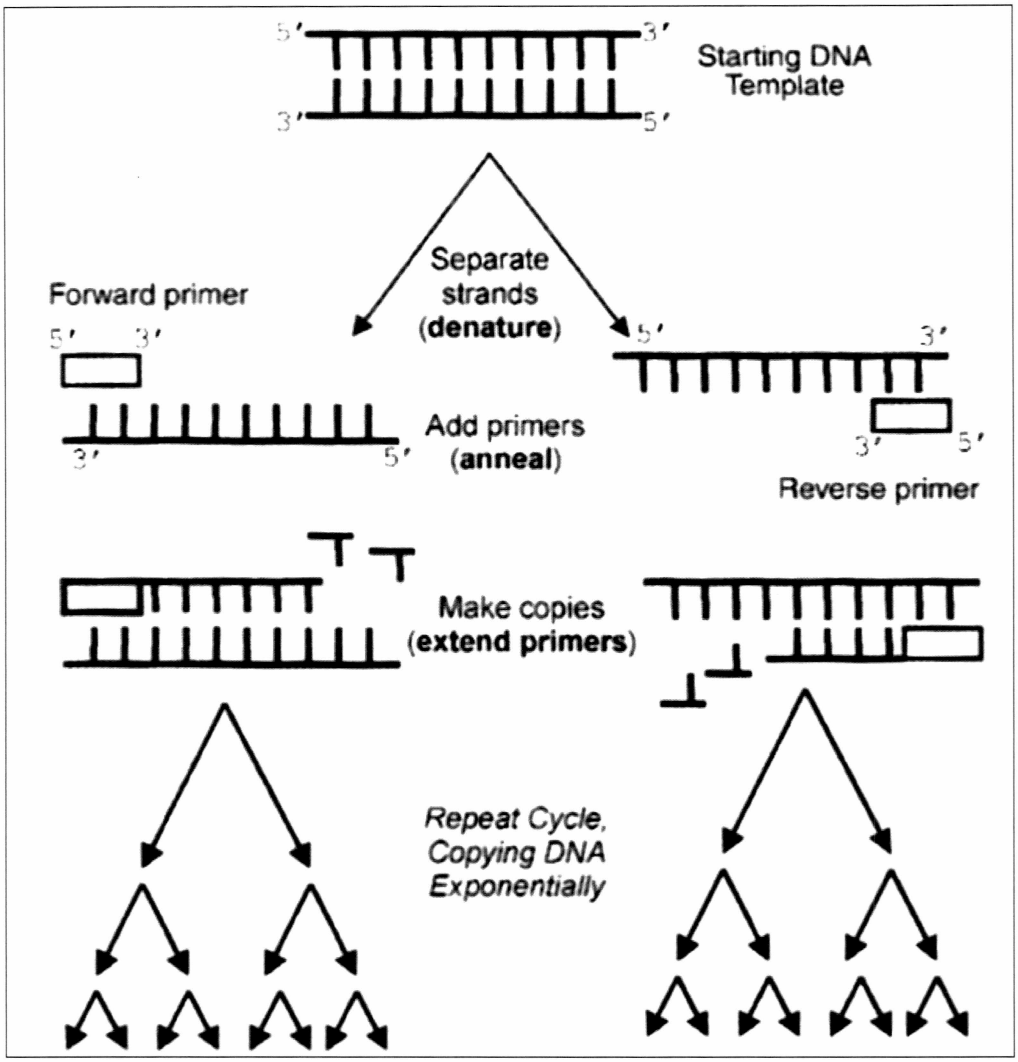

Figure 1.8. In the PCR process, the DNA template strands are denatured, primers are annealed to the resulting single strands, and then the complementary nucleotides are added to the strands to produce a copy of the DNA. This process is repeated many times so it yields many copies of the DNA template. [from Butler, 2001]

In order for the Taq polymerase to start building a new strand of DNA during the second stage of PCR, annealing, it must have a section of DNA as a starting point. A primer is used to start the process. A primer is a short, 15 to 30 base-pairs long, piece of DNA which will anneal, or stick, to the DNA template strand where it finds a sequence complementary to its own sequence [Saferstein, 2002]. Scientists use specific primers which will find sequences that "flank" the portion of the DNA they are interested in amplifying (Figure 1.9).

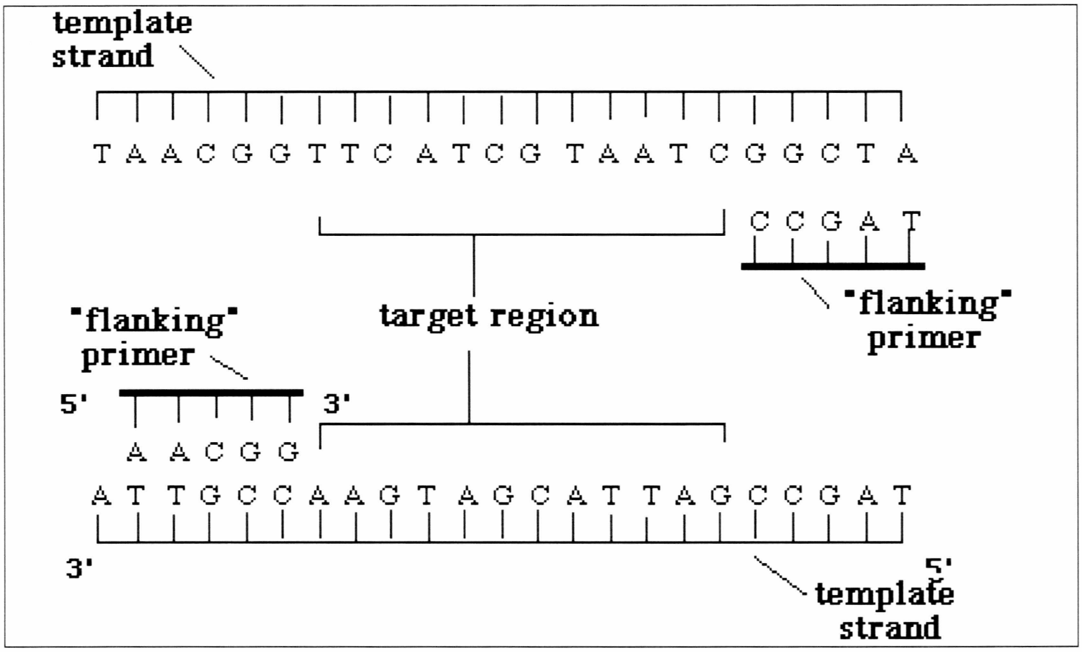

Figure 1.9. The flanking primers bind to the template DNA. [Warder, 1997]

In order for the primer to anneal to the template strand, the temperature must be lowered to a temperature which will allow it to stick well enough to start making a new strand. Primer length and sequence are of critical importance in determining the temperature required for a successful amplification. A simple formula for calculation of the melting temperature (Tm) is: Tm (^{o}C) = 4(G + C) + 2(A + T), where G, C, A, and T are the number of times those bases appear in the primer. Therefore, the annealing temperature chosen for a PCR depends directly on length and composition of the primers. An annealing temperature about 5 ^{o}C below the lowest Tm of the pair of primers in the mixture should be used [Innis and Gelfand, 1990].

A consequence of having too low an annealing temperature is that one or both primers will anneal to sequences other than the true target. Internal single-base mismatches or partial annealing may be tolerated if one wishes to amplify similar or related targets; however, it can lead to "non-specific" amplification and consequent reduction in yield of the desired product. A consequence of too high an annealing temperature is that too little product will be made, as the likelihood of primer annealing is reduced. [Coyne *et al.*, 2002].

During the extension step of the PCR cycle, the Taq polymerase will extend the primer by bringing in complementary nucleotides and attaching them to the template strand. The Taq polymerase works best at temperatures between 72-75°C so the temperature of the PCR mix is raised so that the enzyme can work efficiently. [Innis and Gelfand, 1990]

When these three steps are repeated over and over again, the newly made strands are separated by heat and then go on to serve as template strands for yet more new strands to be made. In this way, the number of double-stranded fragments of DNA potentially doubles during each cycle until there is enough DNA to separate by gel electrophoresis (see Figure 1.10). The number of cycles used for PCR amplification ranges from 20 - 40 cycles [James, 2002].

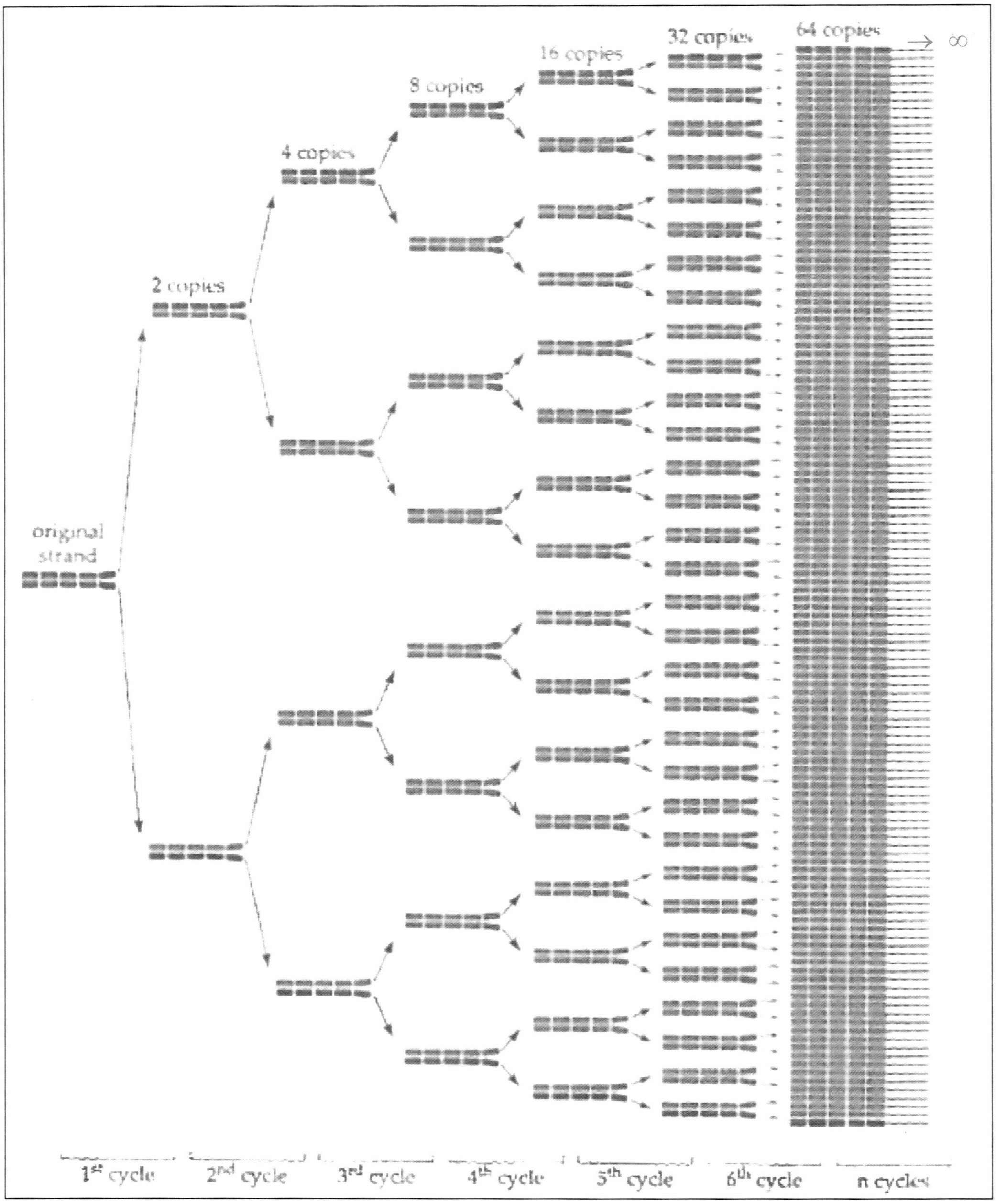

Figure 1.10. The number of copies of the DNA template grows exponentially during PCR according to the number of cycles used [from James and Nordby, 2002].

1.4 Gel Electrophoresis

Once the DNA sample has been amplified by PCR, it undergoes electrophoresis to separate DNA fragments with different lengths. The process of electrophoresis is defined as 'the differential movement or migration of ions by attraction or repulsion in an electric field' [The American Heritage® Dictionary of the English Language, 2000]. In practical terms, a positive (anode) and negative (cathode) electrode are placed in a solution containing ions. Then, when a voltage is applied across the electrodes, solute ions of different charges will move through the solution towards the electrode of opposite charge.

The gel electrophoresis chambers used for the separation of DNA have a positively charged terminal at one end and a negatively charged terminal at the other end. These terminals are connected to a power supply which applies a voltage difference across the gel placed in the chamber. The gel contains sample wells designed to hold the DNA samples being separated. The wells are placed near the negative terminal since the sugar-phosphate backbone of DNA has a negative charge in neutral to basic solutions and will move away from the negative terminal. Once the DNA samples are loaded into the wells, a voltage difference applied across the electrophoresis chamber will cause the sample DNA to move through the gel's pores from the negative (cathode) pole to the positive (anode) pole. The DNA molecules are separated by size because the smaller molecules can move through the pores in the gel faster than larger ones [Butler, 2001].

Agarose is a polysaccharide extracted from seaweed that forms the matrix used in electrophoresis. Purified agarose is in a powdered form and is insoluble in water (or buffer) at room temperature. However, it dissolves in boiling water, so the gel solution is formed by dissolving agarose in boiling water. When the agarose solution starts to cool, the sugar polymers crosslink with each other, transforming the solution in to a semi-solid matrix called a gel. Higher concentrations of agarose in the gel facilitate the separation of small DNA segments, while low agarose concentrations provide greater resolution of larger DNA segments. This is due to the fact that as the concentration of agarose in the gel increases, the pore size of the gel decreases. [Butler, 2001]

To put the DNA samples to be separated into the agarose gel, sample wells designed to hold the samples must be created in the gel. The sample wells are formed in the agarose gel by inserting a plastic comb into the warm gel suspension before the gel has set (see Figure 1.11). This comb is removed once the gel has set producing a number of wells into which individual samples may be loaded. This allows side-by-side separation of a number of samples under identical conditions, which facilitates direct comparison between them.

The agarose gel with small wells formed at one end is placed in the electrophoresis chamber and just covered with a buffer solution (see Figure 1.11). The buffer solution keeps the samples and gel from becoming acidic. [Butler, 2001]

A DNA specific dye, ethidium bromide, is added to the agarose solution to help visualize the DNA bands under ultraviolet light. Ethidium bromide is a fluorescent dye that inserts itself between the bases of nucleic acids and facilitates the detection of DNA fragments in gels. [Rat Genome Database, 2004].

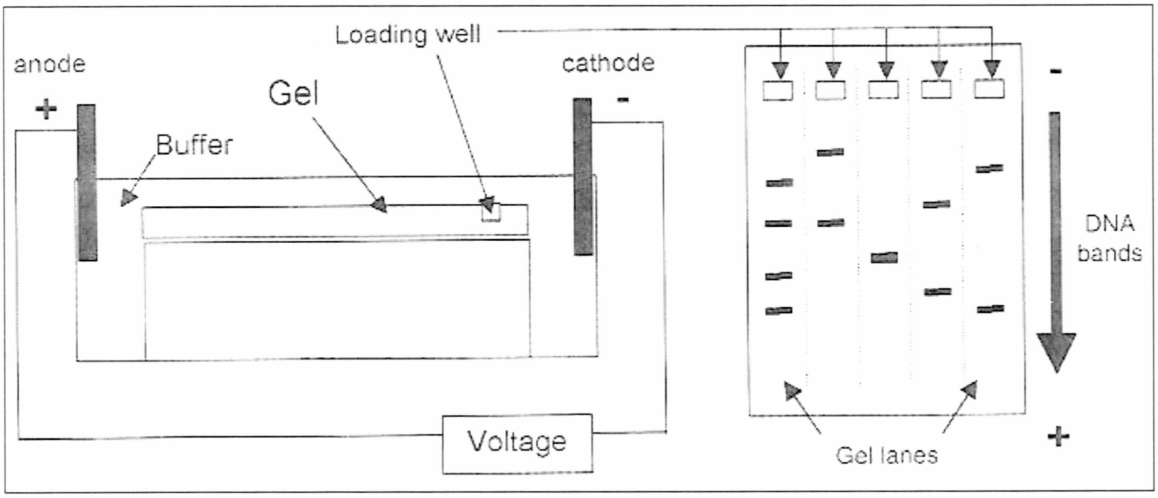

Figure 1.11. An electrophoresis apparatus consists of a chamber with two deep ends and a base for the agarose gel. The electrophoresis apparatus chamber has positive and negative side. The electrophoresis chamber is filled with a buffer solution. Loading wells on the gel create the lanes for DNA migration. [from Butler, 2001]

A loading dye is added to the DNA samples to make placing the samples on the gel easier. The loading dye also helps gauge the distance the DNA has migrated on the gel so the migration can be stopped before the DNA migrates off of the end of the gel. [Butler, 2001]

1.5. Crime Scene Investigations: Blood and DNA Evidence

An increasingly important aspect of criminal investigations is the recovery of biological evidence at crime scenes. Biological evidence includes, but is not limited to, blood, semen, saliva, and hair [James and Nordby, 2002]. The types of biological evidence and where they may be found at crime scenes are discussed in Table 1.1. This study focuses on biological, specifically blood, evidence.

Table 1.1. DNA can be recovered from many types of evidence collected at crime scenes. [adapted from Saferstein, 2002]

Evidence	Possible Location of DNA on the Evidence	Source of DNA
baseball bat or similar	Handle, end	sweat, skin, blood, tissue
hat, bandana, or mask	Inside	sweat, hair, dandruff
eyeglasses	Nose or ear pieces, lens	sweat, skin
facial tissue, cotton swab	Surface area	mucus, blood, sweat, semen, ear wax
dirty laundry	Surface area	blood, sweat, semen
toothpick	Tips	saliva
used cigarette	Cigarette butt	saliva
stamp or envelope	Licked area	saliva
tape or ligature	Inside/outside surface	skin, sweat
bottle, can, or glass	Sides, mouthpiece	saliva, sweat
used condom	Inside/outside surface	semen, vaginal or rectal cells
blanket, pillow, sheet	surface area	sweat, hair, semen, urine, saliva
"through and through" bullet	outside surface	blood, tissue
bite mark	person's skin or clothing	saliva
fingernails	Scrapings	blood, sweat, tissue

Recognition and identification of biological evidence is a crucial process in investigations (see figure 1.12). Once an investigator has identified an item as evidence, whether it is biological, chemical or physical, that item must be compared with controls and standards of similar nature. This study identified the evidence, the blood on the substrates, as blood by using a chemical treatment on the questioned area.

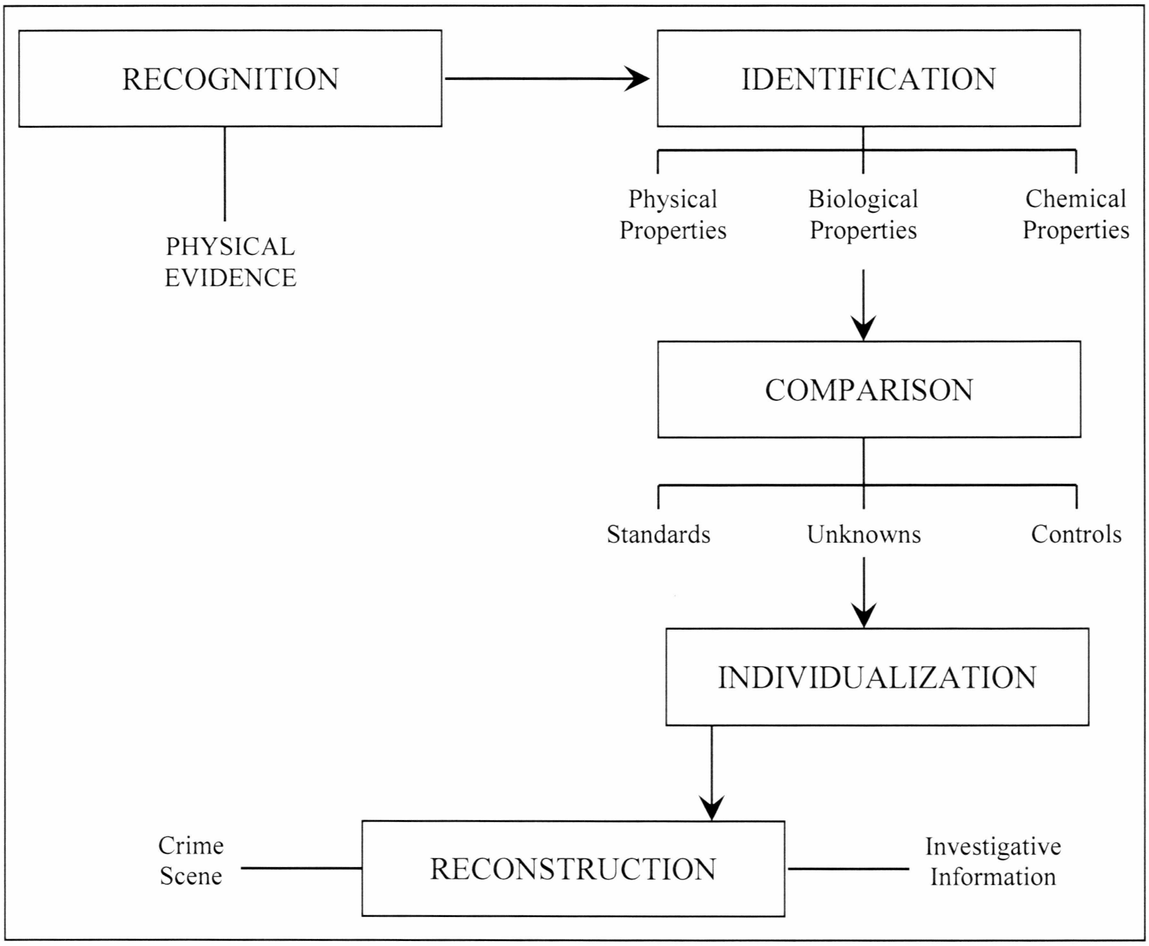

Figure 1.12. The process of forensic examinations at crime scenes follows a specific principle of recognition, identification, comparison, individualization, and reconstruction. [adapted from Lee, 2001]

The connection between evidence collected at a crime scene and the individuals involved with that crime is called the linkage theory. The linkage theory is based on the idea that everything involved with a crime can be linked to one another in some way (illustrated in Figure 1.13).

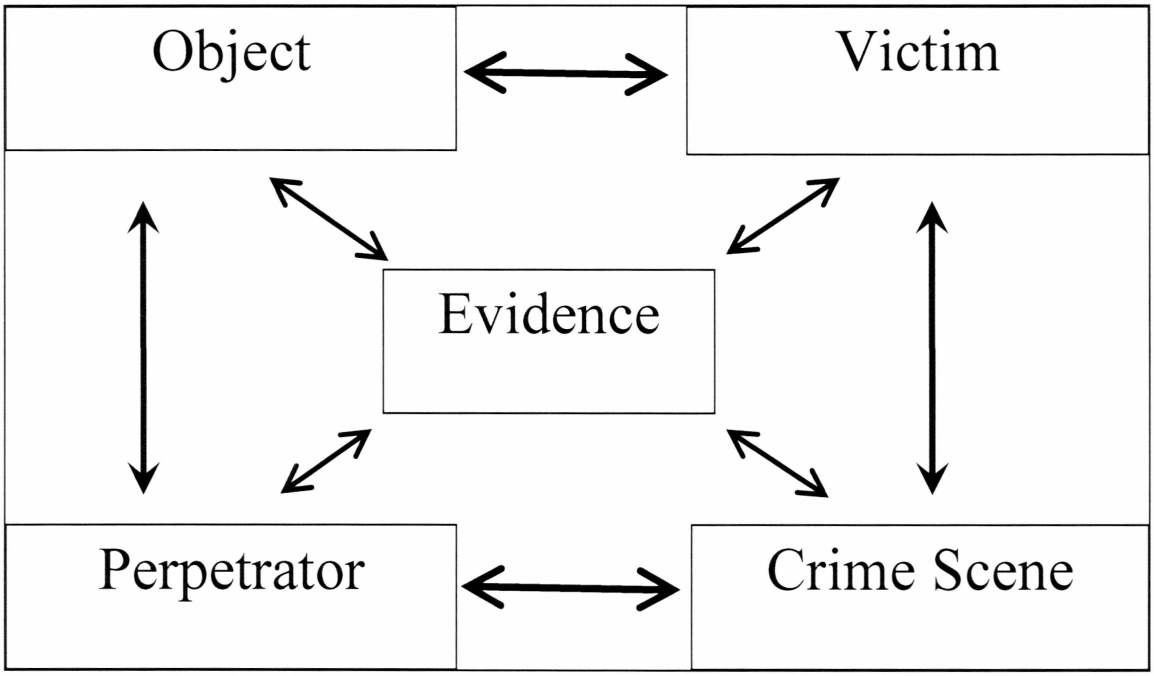

Figure 1.13. The picture shows a visual representation of the linkage theory. [adapted from Lee, 2001]

1.5.1. Role of Blood and DNA Evidence

Blood evidence associated with a crime can provide information that may solve the case, so it is essential to correctly document, collect, and preserve this type of evidence. The term 'blood evidence' refers to the blood and any article in contact with the blood [James and Nordby, 2002]. Properly collected and preserved blood evidence can establish a strong link between an individual and a criminal act. Improperly handled blood evidence can weaken or destroy a potential source of facts in a case by introducing doubt as to the veracity of the blood evidence results. Blood evidence or the lack of blood evidence can also be used to bolster or contradict a witness statement or any statements that the suspect may make. Blood evidence can also point the investigator in the direction he or she needs to go to solve the case. If blood evidence is documented, collected, stored and analyzed suitably, it can be presented to a judge or jury several years from the time of the criminal act.

One of the most powerful applications of blood evidence is the ability to eliminate a person as a potential suspect in a crime. A blood source can now be statistically narrowed down to one person out of several million or even several billion through DNA sequencing [Shiro, 2004].

DNA sequencing is the process in which restriction enzymes cut the very long DNA strands found in cells and viruses into discrete, reproducible fragments with unique nucleotide sequences [James and Nordby, 2002]. This helps scientists quickly identify known nucleotide sequences within larger DNA fragments. These unique sequences are then compared to similar sequences obtained from other DNA to identify the source of the DNA of interest.

One of the most commonly used techniques for determining the nucleotide sequence of a DNA fragment involves using a modified PCR process and then separating the new DNA strands by gel electrophoresis. [James and Nordby, 2002] In this method, a mixture containing the DNA fragment of interest is divided into four portions. Each portion contains all the components (Taq polymerase, dNTPs, buffers, and primers) required for the synthesis of complementary DNA strands. In addition, each portion contains a modified form of one of the four different nucleotides. A dideoxynucleotide triphosphate, ddNTP, has the same structure as a dNTP, but contains a hydrogen atom instead of a hydroxyl group at its 3' end (see Figure 1.14 (A)). This prevents it from participating in further extension. Therefore, when a ddNTP is incorporated into the DNA, further chain elongation is blocked. This results in a population of truncated DNA strands of varying lengths (shown in Figure 1.14 (B)). These extension fragments begin at the end of the primer and extend in the 3' direction until a ddNTP is incorporated in the strand. There are four different ddNTPs that correspond to the four DNA nucleotides, and each ddNTP has a different color. When separated by electrophoresis, a "ladder" of these truncated DNA strands will form, each containing a fluorescently labeled ddNTP at its 3' end. The sequencing ladder appearing in the four lanes of the electrophoresis gel will be composed of colored bands caused by the ddNTPs (shown in Figure 1.14 (C)). The sequence of nucleotides can then be determined by correlating the color of a band on the gel with its specific ddNTP, and the order in which the bands appear on the gel (see Figure 1.14 (D)). [Saferstein, 2002]

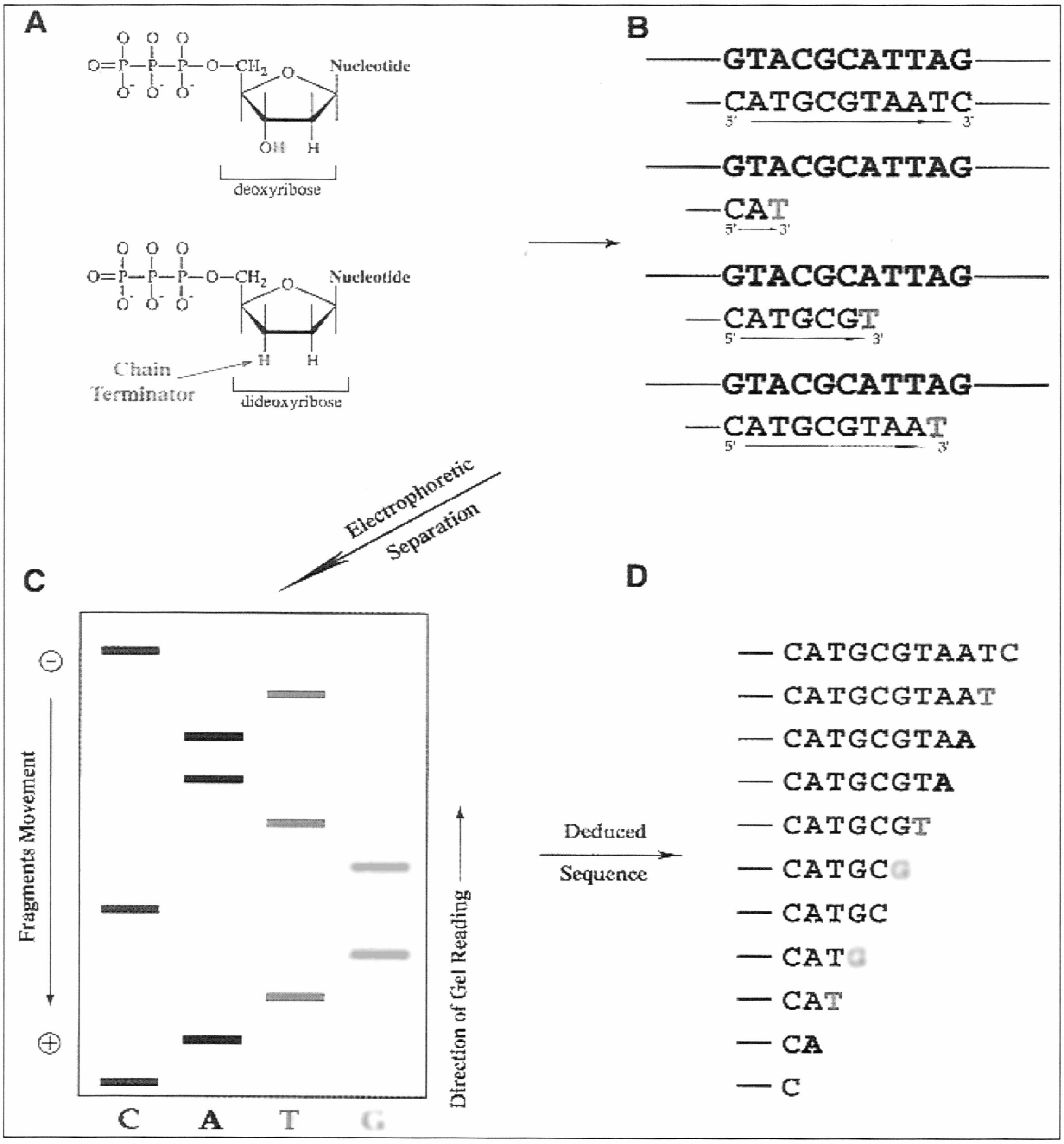

Figure 1.14. A and B show the nucleotide sequence of DNA may be determined by including a small percentage of dideoxynucleotide chain terminators for one deoxynucleotide in the DNA replication mix. When this chain terminator is incorporated into a growing chain, further elongation is blocked. Part C shows that the terminated nucleotide chains of different lengths are separated on the electrophoresis gel with shorter fragments at the bottom of the gel and larger fragments at the top of the gel. Part D shows how the sequence of nucleotides can be read from the bottom of the gel up to the top of the gel. [from James and Nordby, 2002]

The position of a band across the width of a gel indicates the base nucleotide that terminated that fragment. Thus, the sequence of the bands down the length of the gel is determined by the sequence of the bases within the DNA fragment. The sequence of the original DNA strand is then deduced from the complementary sequence of the new strand (see Figure 1.14 (D)). This unique sequence is what investigators use to identify the person associated with the blood evidence. However, the investigators must be able to find the blood before they can sequence the DNA and identify the person(s) associated with the blood evidence. [James and Nordby, 2002]

1.5.2. Luminol

Luminol is one of the most commonly used presumptive bloodstain detection reagents. Luminol, 3-aminophthalhydrazide (the structure is shown in Figure 1.15), is an organic compound which, when oxidized, emits light [Bassam, 1983]. This phenomenon is known as chemiluminescence and lasts for approximately 30 seconds for luminol [Hesskew, 1991].

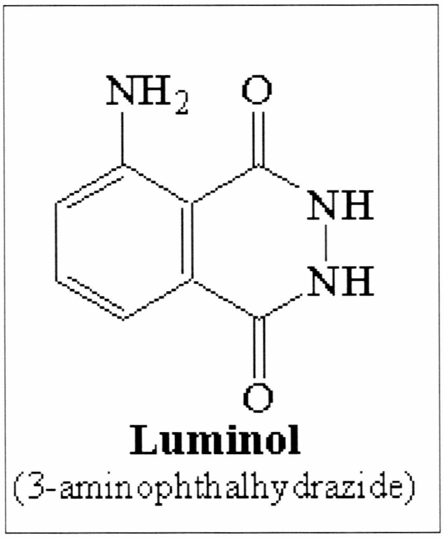

Figure 1.15. Luminol is an organic compound also known as 3-aminophthalhydrazide [from Bassam, 1983].

Luminol is applied to suspected bloodstains using a pump or aerosol sprayer. Luminol oxidation is catalyzed by the presence of a metal ion, such as iron (II) (see the mechanism is Figure 1.16). The iron in the heme groups of the hemoglobin in red blood cells oxidizes the luminol and causes the blood to 'glow' in the dark (see Figure 1.17). [James and Nordby, 2002]

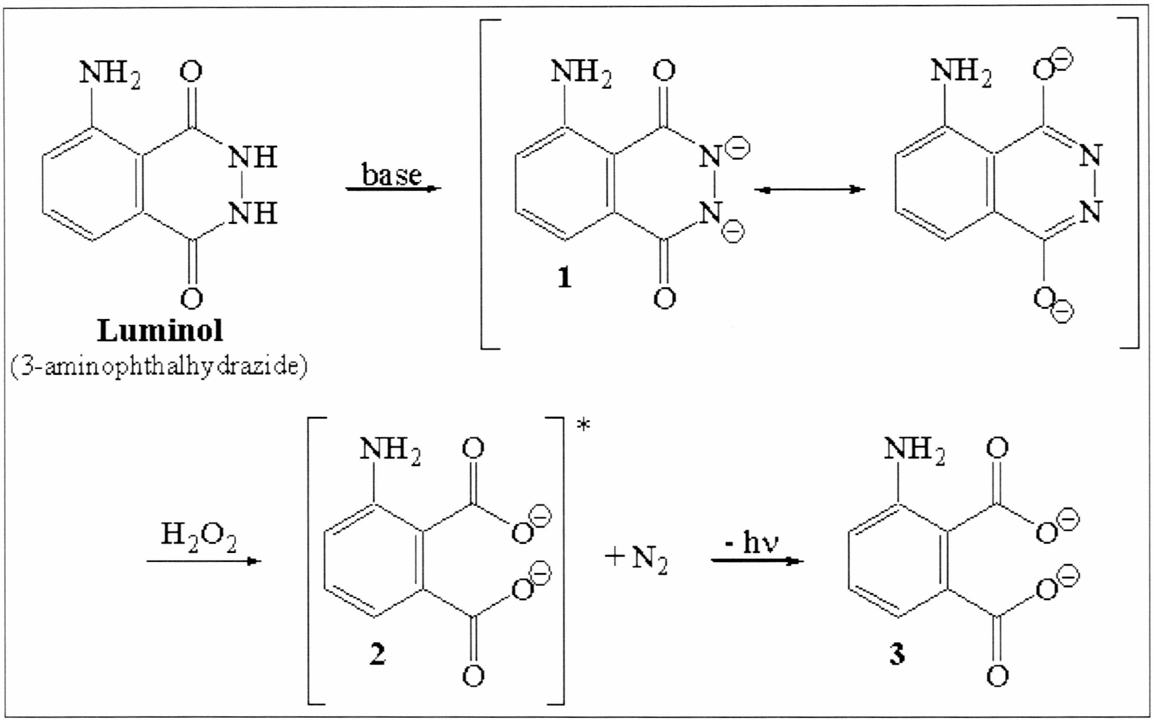

Figure 1.16. The luminol is converted by the basic solution into the resonance-stabilized dianion (**1**). Then it is oxidized by the hydrogen peroxide into the dicarboxylate ion (**2**) and molecular nitrogen, N_2. When the molecule (**2**) is formed, it is in an excited (higher energy) electronic state. It sheds its "extra" energy by emitting a photon of light (hν), which allows the molecule to go to its ground state form (**3**). [from Bassam, 1983]

Figure 1.17. The picture shows a simulation of luminol at work. Before spraying luminol there is no sign of blood, but after spraying luminol, the latent blood traces emit a blue glow. [from How Stuff Works, 2002]

Some crime scene investigators use luminol as their first choice for detecting blood. However, by using luminol in a reckless manner, it is possible to lose valuable information from a bloodstain so alternative methods of identifying blood should be attempted initially. An alternative light source (ALS), typically UV light, should be used first when searching for blood at a crime scene. Blood will quench the fluorescence when UV light is applied so it will appear dark compared to its surroundings [Saferstein, 2002]. Blood has a tendency to flow into floorboard cracks, into carpet padding, behind baseboards, etc., so by conducting a thorough examination with an ALS; the investigator can often find these hidden stains. The item containing the blood can then be collected and submitted to the crime lab for confirmatory testing. [Shiro, 2004]

Luminol has several drawbacks as a presumptive test for blood, so using luminol at a crime scene should be carefully evaluated. The problems with luminol include: (1) the luminol reaction is a presumptive test for blood. If the stain is so dilute that it can only be visualized with luminol, then no further analyses can be performed to confirm the presence of blood [Shiro, 2004]. (2) Luminol can give false positives. Luminol will react with copper compounds, iron compounds, cobalt ions, potassium permanganate (found in some dyes), hydrated sodium hypochlorite (bleach), and plant peroxidases which are all common oxidizing agents [James and Nordby, 2002; Shiro, 2004]. (3) Studies have shown that luminol will cause the loss of several genetic markers. Luminol could also further dilute an already diluted stain beyond the genetic marker analysis detection limits [Shiro, 2004]. (4) Luminol is water-based so it could possibly cause bloody impressions to smear, making identification of fingerprints or footprints impossible [Shiro, 2004]. (5) The short duration of luminescence makes the blood difficult to document [James and Nordby, 2002]. (6) Luminol does not require the use of an ALS; however, the reaction can only be viewed in an area that is completely dark. This is very difficult to accomplish for outdoor crime scenes.

In addition to the technical and practical drawbacks of luminol, luminol has been listed in some literature as banned for use in California. The current Material Safety Data Sheet (MSDS) lists luminol as "possibly carcinogenic." Therefore, finding a safe, reliable alternative bloodstain enhancement technique would be very valuable. [Cheeseman, 1995]

1.6 Fluorescein

Fluorescein, a chemical (shown in Figure 1.18 and described in Table 1.2) that has recently begun to be used as an alternative to luminol, is an extremely useful reagent for the detection and enhancement of suspected bloodstains, particularly on multi-colored backgrounds.

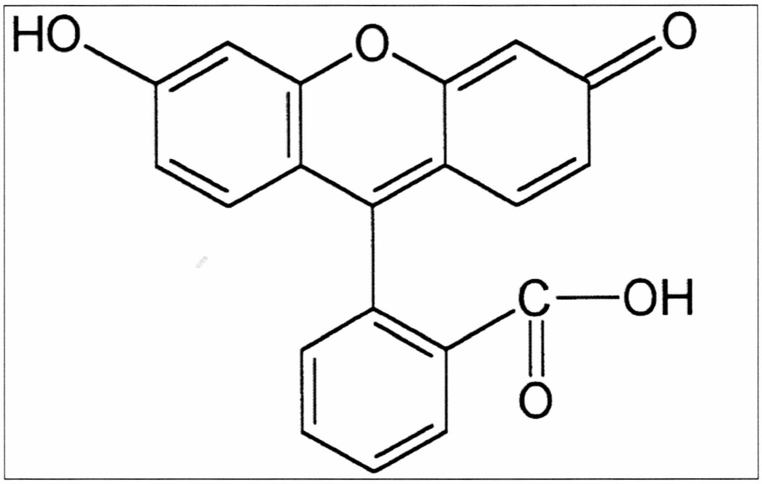

Figure 1.18. Fluorescein has the structure shown above. [from James and Nordby, 2002]

Fluorescein is converted, by the catalytic action of hemoglobin, into a dianion form that emits a bright yellow/green fluorescence. After application of the fluorescein and a catalyst by spraying, the fluorescein-treated bloodstains are visualized with blue light and orange safety glasses. The stains will remain fluorescent for 15-30 minutes without repeated applications of the fluorescein, allowing enough time for the stain or pattern to be photographically documented. The new, solvent-based formulation of fluorescein being used at crime scenes is more stable, has a longer shelf life, and is less hazardous than luminol. The fluorescein detection method offers the advantage of working in a lighted environment, and the fluorescence persists longer than that of luminol. [Patonay *et al.*, 2002]

Table 1.2. The MSDS for fluorescein provides the properties of the compound [from Sigma Chemical Co, 1999].

Synonyms	soap yellow
Molecular formula	$C_{20}H_{12}O_5$
Molecular Weight	332 g/mol
Appearance	orange-red crystalline powder
Melting point	315 °C
Water solubility	Slight (producing a weakly green-yellow solution)
Toxicology	May act as an irritant.
Excitation wavelength	494 nm
Emission wavelength	520 nm

1.6.1. History

Although fluorescein, like luminol, is listed as "possibly carcinogenic" on its MSDS, it has been used in medicine since the 1940s. More recently it has been the angiography diagnostic tool for vascular ophthalmic disorders requiring FDA approval for clinical use [Cheeseman, 1995].

Fluorescein is used to label molecules for detection in capillary electrophoresis and to label primers used in automated DNA sequencing [Sjoback *et al.*, 1998].

1.6.2. Structure and Fluorescence

The conjugation of the bonds in the fluorescein compound, shown in Figure 1.18, leads to its ability to fluoresce. Conjugation is the alternating of double bonds (π bonds) in a molecule.

Oxidation of fluorescin to fluorescein yields a yellow/green fluorescence. Oxidation is a reaction in which a decrease in electrons by carbon, either by bond formation between carbon and a more electronegative atom (usually oxygen, nitrogen, or a halogen) or by bond breaking between carbon and a less electronegative atom (usually hydrogen) [McMurry, 1999].

Fluorescein exists in several forms (see Figure 1.19). The monoanion absorbs at 453 and 472 nm and has moderate fluorescence, while the dianion absorbs at 490 nm and is intensively fluorescent [Smith and Pretorius, 2002]. It is the dianion form that is produced during oxidation and yields the yellow/green fluorescence.

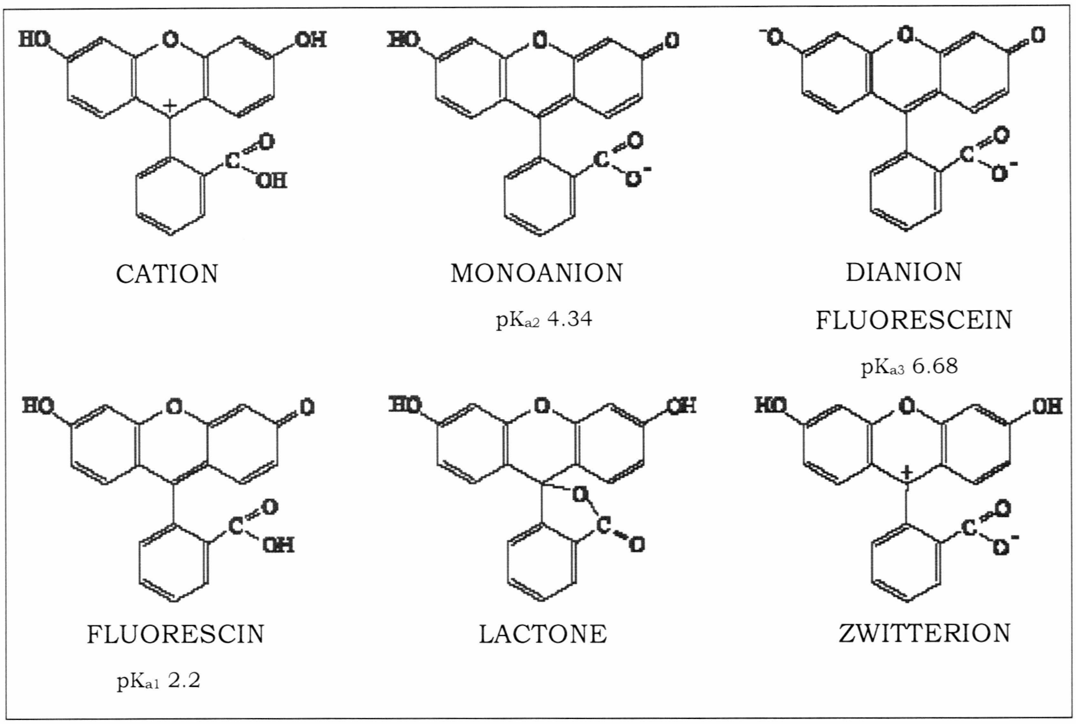

Figure 1.19. The six ionic forms of fluorescein are shown above. [from Zanker and Peter, 1958]

The blue light is used to excite the fluorescein molecule. Orange filtered glasses are used to view the fluorescein because it blocks out the blue light being emitted. This allows any fluorescence to be seen. The visible color is the opposite (complementary) of the color being absorbed [Merritt, 2001].

1.6.3 pH Dependence

The fluorescence and absorbance of fluorescein is pH sensitive, with both processes being the most efficient at pH 8. Table 1.3 illustrates the changes in absorbance and fluorescence caused by varying the pH of the fluorescein solution [Fisher, 2004].

Smith and Pretorius (2002) determined the pKa for three forms of fluorescein (as shown in Figure 1.21): neutral (the quinonoid according to Figure 1.21) with a pK_{a1} of 2.22, the monoanion with a pK_{a2} of 4.34, and the dianion with a pK_{a3} of 6.68.

Table 1.3. Fluorescein absorbance and fluorescence are affected by varying pH. [Fisher, 2004]

pH	Absorbance %	Fluorescence %
8.0	98	96
7.0	85	84
6.5	66	57
6.0	50	32
5.0	36	11

The fluorescent properties of fluorescein dramatically change at low pH. Fluorescein has a pKa of 6.68 and shifts to its protonated form in low pH solutions. The protonated form is poorly fluorescent. Therefore, all studies done using fluorescein should be done at pH 7.0 or higher. [Integrated DNA Technologies, 2005]

1.6.4. Interactions and Similar Compounds

Fluorescein is a presumptive searching technique for latent blood, yet it is subject to false positives similar to those experienced by investigators using luminol and phenolphthalein such as copper compounds, iron compounds, cobalt ions, potassium permanganate (found in some dyes), hydrated sodium hypochlorite (bleach), and plant peroxidases.

Fluorescein binds tightly in an aromatic slot [Omelyanenko *et al.*, 1993]. Although the actual mechanism of the reaction with fluorescein and blood is not known, it is presumed to be similar to reaction of phenolphthalein and blood shown in Figure 1.20.

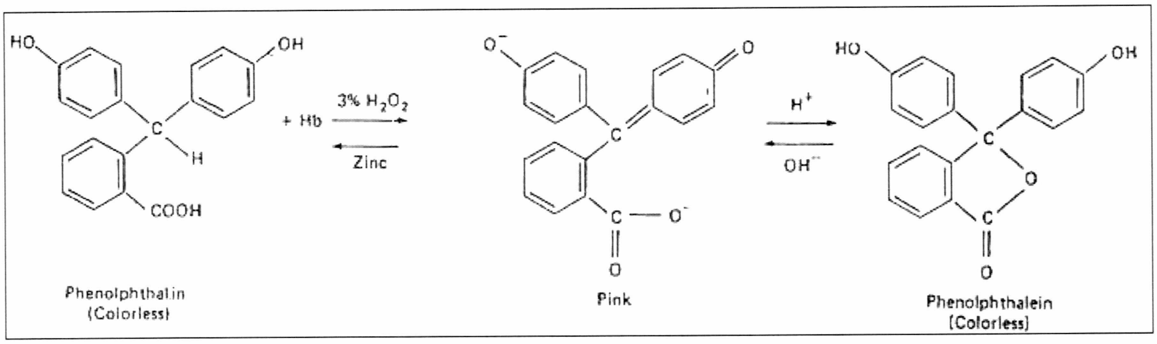

Figure 1.20. The chemical used in a presumptive blood test, phenolphthalein, has a similar structure to fluorescein. [adapted from Purvis, 2005]

Phenolphthalein in basic conditions generates a deep pink-colored species. This is then reduced over zinc metal to produce colorless phenolphthalin. It is this reagent that is used in the presumptive test for blood. Addition of an aqueous solution of phenolphthalin to blood produces no color change until the hydrogen peroxide is added. The reduced phenolphthalin is rapidly oxidized by hydrogen peroxide to the deeply pink-colored phenolphthalein as shown in the Figure 1.20. [Purvis, 2005]

1.6.5. Fluorescein Usage at Crime Scenes

Cheeseman (1995) claims fluorescein should be used to identify bloodstains when the blood concentration of the sample has been so diluted that it can no longer be

seen by any other means, and/or the surface on which the stain is located lacks adequate color contrast differential to easily spot the blood stain. If any whole bloodstains are found, adequate samples should be taken for further serological testing before the fluorescein technique is employed to prevent the risk of the fluorescein destroying the genetic material. Cheeseman further explains that the surface texture of the substrate upon which the stain is located also plays an important role. Porous versus non-porous surfaces and vertical versus horizontal factors are important to the success of this technique. These factors can prove to be problematic as in the case where a non-porous substrate on a vertical surface where application can cause running of the stain.
Prior to use in the field, the fluorescein solution used to identify the latent bloodstains, called the working solution, must be prepared. The fluorescein working solution is made from the fluorescein stock solution and ethanol. The fluorescein stock solution consists of 0.1 g fluorescein, mixed with 20 mL ethanol, 1 mL glacial acetic acid and 2.0 g of zinc. The zinc and acetic acid keep the fluorescein in its reduced form. The fluorescein working solution is then prepared by decanting 1 mL of the stock fluorescein solution off the zinc and then diluting it in 99 mL ethanol. The working solution is best used within three hours. Through personal experience, the working solution is still usable after three hours; however, the longer the working solution is exposed to air, the less of an impact it will have for the detection of bloodstains. [Cheeseman, 1995; Marie *et al.*, 1998; Martin, 2002]

Application of the working solution of fluorescein is conducted as follows. The working solution is placed in a spray bottle and is used to spray the area of interest. The area is misted with a fine, uniform spray of the solution from a distance of 12"-18" away from the surface. A light and even mist yields the most successful results. However, non-porous vertical surfaces are susceptible to reagent running, which can distort or destroy any bloodstain patterns. Therefore the use of the commercial thickener, such as Keltrol RD, is recommended in all circumstances [Cheeseman, 1995]. The catalyst, hydrogen peroxide (3%), is applied in the same manner described above. The hydrogen peroxide oxidizes the fluorescein-heme complex and promotes the fluorescence. The area is then viewed with blue light (around 490 nm) using orange safety goggles. [Cheeseman, 1995; Marie *et al.*, 1998; Martin, 2002]

Application of the working solution on the targeted area will produce a yellow-green color to appear within a few seconds if blood is present. At this stage, the bloodstain will be apparent and some background colorization may also be apparent. However, the application of the hydrogen peroxide will help to reduce the background interference (shown in Figure 1.21 (c) and (d)) by enhancing the reaction between fluorescein and blood, clarifying the bloodstain pattern. The bloodstained areas will fluoresce when illuminated by a blue light source (see Figure 1.21).

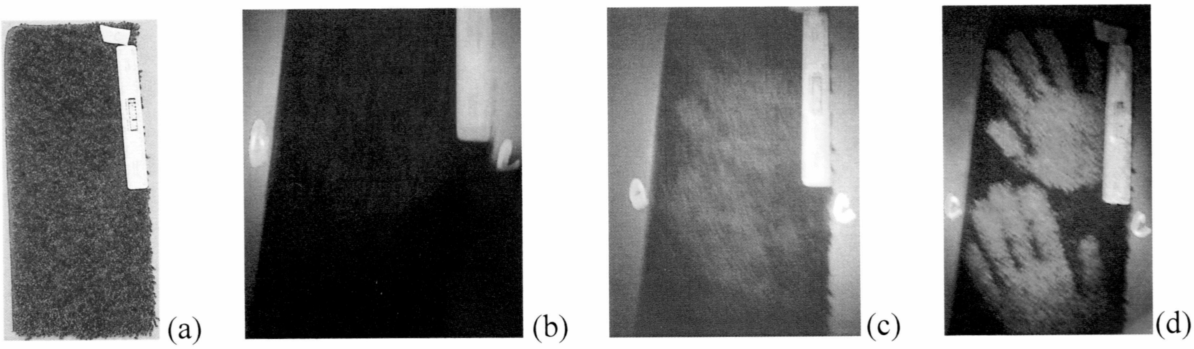

Figure 1.21. The first picture (a) is of a dark piece of carpet containing latent blood prints. (b) shows a visualization of the carpet using blue light. (c) shows the carpet after fluorescein is applied to the carpet and the carpet is visualized using blue light. (d) shows the carpet under blue light after hydrogen peroxide was applied to catalyze the fluorescence [from Martin, 2002].

The purpose of this technique is to enhance the appearance of latent bloodstain patterns. It will not differentiate the type, or source, of the bloodstain and can yield false positive results with certain substances (i.e. Fe, Cu, or soil). A positive (known blood) and a negative (water) control should be simultaneously performed during routine examination to examine the effectiveness of the current working solution.

The technique of using fluorescein to identify latent bloodstains at crime scenes is proving to be very useful and would be even more useful if DNA could be recovered from the stains identified this way. So the purpose of this thesis was to determine if DNA could be recovered from denim substrates containing latent blood after treating the substrates with fluorescein.

Chapter 2

EXPERIMENTAL METHODS

The experiment was designed to determine if DNA could be recovered from blood stained denim substrates after using fluorescein to identify the blood. The experimental methods involved applying blood to the denim substrates, drying the substrates, treating the substrates, extracting the DNA from the blood-stained denim using a chelex solution, amplifying the extracted DNA using PCR, separating the DNA fragments by gel electrophoresis, and visualizing the DNA fragments using UV light. These methods are discussed in detail in the following sections of the chapter.

2.1. Substrates

The substrates used for the experiment were pieces of blue denim jeans (Levi jeans). Denim jeans were chosen for testing since they are commonly found in households and often collected as evidence from violent crime scenes.

The denim jeans were washed in clean water in a regular clothes washer, dried, and then cut into 1.5 ± 0.01 square-inch portions. All the denim portions were evaluated with a blue light (Emissive Energy, blue LED light, X5MT-BB) and orange filtered safety glasses (Smith & Wesson, Magnum 3G Z87) prior to any testing in order to rule out any fluorescence due to other sources (such as natural fibers, detergents, etc.).

2.2. Blood Samples

Six different dilutions of blood in deionized (DI) water were added to the substrates or chelex solutions using three different methods of treating the samples (Table 2.1). The blood dilutions were prepared by diluting whole blood from the researcher with deionized water (DI) to produce a range of blood concentrations between 1: 1 to 1: 50,000 [parts blood to parts DI water as determined by volume (mL ± 0.1)]. The range of dilutions is shown in Table 2.1. The range of dilutions was chosen to simulate how diluted blood may become if an area or substrate is cleaned in an attempt to remove evidence. Deionized (DI) water was used as the negative DNA control.

Table 2.1. The experimental design includes three methods for testing the recovery of DNA from a blood sample. A (+) in the table indicates that DNA was extracted from the sample while a (–) in the table indicates that no DNA was extracted from that sample. The sample size was 21 and the experiment was performed twice.

		Blood Dilutions (Parts Blood to Parts DI Water)						
Method	**Substrates**	1 : 1	1 : 10	1 : 100	1 : 1000	1 : 10000	1 : 50000	DI Water (control)
(a) Not Treated with Fluorescein	NA (*direct*)	+	+	+	+	+	+	-
(b) Not Treated with Fluorescein	Denim	+	+	+	+	+	+	-
(c) Treated with Fluorescein	Denim	+	+	+	+	+	+	-

The blood samples were subjected to three different treatment methods. In the first method, (a), diluted blood was directly applied to a chelex solution in 40 ± 0.1 μL aliquots using a micropipette (Labnet, Model P3945-200). In the second method, (b), diluted blood was applied to the center of the denim substrates in 40 ± 0.1 μL aliquots using a micropipette. The blood sample formed a spot approximately 4 ± 0.1 mm in diameter on the substrate. The samples were then allowed to dry for 3 ± 0.1 days in the laboratory at ambient room temperature (23 ± 1 °C). The dried blood stains on the denim were not visible beyond the 1:10 dilution of blood. In the third method, (c), method (b)'s steps were repeated except that after the blood dried, it was treated with fluorescein and hydrogen peroxide before the DNA was extracted from the substrate. The fluorescein was applied using a spray pump bottle in a light spray (0.20 ± 0.01 mL) that just covered the substrate. Then the hydrogen peroxide was applied in the same manner as the fluorescein. The blood stains were checked for fluorescence immediately after the fluorescein treatment using the blue light and orange filtered glasses. All the substrates had a visible fluorescence in the location where the blood was applied. After observing the fluorescence, the blood stains were extracted.

2.3. DNA Extraction

For the denim samples, sterile scissors and tweezers were used to cut out a 4 ± 0.5 mm by 4 ± 0.5 mm square section of substrate that contained the tested area. The denim squares were placed in 1.5 mL centrifuge tubes. Next, 0.75 ± 0.01 mL of DI water was added to each tube and the sample was incubated at ambient room temperature (23 ± 1 °C) for 30 ± 1 minutes. The tubes were then vortexed using a Vortex Genie2^{TM} (VWR Scientific Products, Model #G560) for 5 ± 1 seconds and centrifuged using a Brinkmann centrifuge (Model # 5415C) for 1 ± 0.1 minute at a maximum speed of 7000 ± 70 revolutions per minute (RPM) [Budowle et al, 1995]. The supernate, approximately 500 ± 10 μL, was drawn off using a micropipette and placed into another 1.5 mL centrifuge tube.

A chelex extraction, as discussed in Chapter 1.3, with a 5% weight per volume chelex solution (5 g Chelex 100^{TM} to 100 mL of DI water) [Budowle et al, 2000] was performed by adding 200 ± 10 μL of the chelex solution to each tube using a micropipette and vortexing each sample gently for 1 ± 0.1 minute [Chelex 100^{TM} was purchased through Bio-Rad Laboratories]. The tubes were then incubated at 56 ± 1 °C for 10 ± 0.1 minutes in a water bath (NESLAB, Model #RTE-111). At the halfway point of the incubation, 5 ± 0.1 minutes, the contents of the tube were remixed by placing them on a vortex stirrer for 15 ± 1 seconds and then placing them back in the 56 ± 1 °C bath for the remaining 5 ± 1 minutes. After the incubation, the contents of the tubes were remixed and then placed in a boiling water bath (approximately 100 ± 1 °C) for 6 ± 0.1 minutes [Waye et al, 1989]. The tubes were again vortexed to re-suspend the contents and then spun down for 5 ± 0.1 minutes in a centrifuge at 7000 ± 70 RPM.

Literature reports that chelex binds to metal ions such as magnesium [Butler et al, 2001]. The chelex settles to the bottom of the tube because it is denser than DNA. This allows the supernate, the DNA and water, to be pipetted off and then used for DNA amplification. For the DNA amplification, 6 ± 0.1 μL of the DNA was pipetted off the top of the centrifuge tube containing the chelex solution.

2.4. Polymerase Chain Reaction

The extracted DNA, the DNA template, was amplified (see Chapter 1.3 for a discussion of amplification) at the D18S51 locus [Comey et al, 1994]. The D18S51 locus was chosen based on its use in other studies and because it is one of the 13 CODIS core loci shown in Figure 1.6.

To perform this amplification, the DNA template, a 6 ± 0.1 μL portion of the upper supernatant phase from the centrifuged sample, was transferred from the sample tube into the PCR tube. Then a 6 ± 0.1 μL portion of the positive DNA control, 9947A, a human cell line DNA, was placed into a clean PCR tube [Budowle et al, 1999]. Lastly, a 6 ± 0.1 μL portion of the negative DNA control, DI water, was also placed into a clean PCR tube.

A 12 ± 0.1 μL portion of the PCR Master Mix (purchased through Promega) was then transferred into each of the PCR tubes. The Master Mix contains all four dNTPs (400 mM each), 50 units/mL of Taq DNA Polymerase [supplied in a proprietary reaction buffer (pH 8.5)], and $MgCl_2$ (3 mM). All four dNTPs (dATP, dGTP, dCTP and dTTP) are required for the synthesis to occur. A discussion of the components of the Master Mix and their impacts on PCR is contained in Chapter 1.3.

The DNA primers (prepared and purchased through Invitrogen™ Life Technologies) were added in 3.5 ± 0.1 μL quantities to each tube just prior to loading the samples into a thermocycler (Perkin Elmer DNA Thermal Cycler, P18282). Two primers are required for PCR: primer 1 (P1) is the forward primer and primer 2 (P2) is the reverse primer. Primers are discussed in detail in Chapter 1.3. The primer sequences were 5'-CAAACCCGACTACCAGAAC-3' for P1 and 5'-GAGCCATGTTCATGCCACTG-3' for P2. The primer sequences used were selected using the FBI CODIS Core STR Loci Fact Sheet [NIST, 2005] (an excerpt of the table is shown in Table 2.2).

Table 2.2. The table shows the portion of FBI CODIS core STR loci fact sheet [NIST, 2005] used for selecting the primers for the D18S51 locus.

PCR Primer Sequences
5' – CAA ACC CGA CTA CCA GCA AC – 3'
5' – GAG CCA TGT TCA TGC CAC TG – 3'

Before the thermocycler was started, mineral oil was added in a 20 ± 1 μL aliquot to each sample to prevent evaporation [Budowle and Moretti, 1999]. The thermocycler was programmed to conduct the following PCR amplification conditions: 40 cycles of 1 minute at 95 °C to denature the DNA followed by 1 minute at 58 °C to anneal the DNA and 2 minutes at 70 °C to extend the DNA. Then the sample underwent 10 minutes at 72 °C to perform the final extension of the DNA. The sample was then maintained at 4 °C for 10 ± 0.5 hours at which time it was subjected to gel electrophoresis. [Promega, 2001]

2.5. Gel Electrophoresis

Gel electrophoresis was used to separate the DNA fragments. The gel used to perform the analysis in this study used a gel with a concentration of 2% agarose as discussed in Chapter 1.4. The gel was prepared by placing 1.6 ± 0.001 g agarose in a 250 mL flask with 80 ± 0.1 mL of 1X TAE (Tris-Acetate-EDTA) buffer solution and 42 ± 0.1 μL of ethidium bromide. The 1X TAE buffer solution was prepared by placing 20 ± 0.1 mL of 50X TAE buffer (purchased from Bio-Rad Laboratories) into a 1 L volumetric flask and filling it to 1000 ± 0.1 mL with DI water. The purposes for TAE buffer and ethidium bromide solutions are discussed in Chapter 1.4. The agarose, buffer and ethidium bromide solution was mixed on a stirring hot plate until the agarose was dissolved and solution was clear. Once cool to the touch, the agarose gel solution was poured into an electrophoresis tray (10 cm x 15 cm) to a depth of 5 ± 0.1 mm. Wells were made in the agarose gel as shown in Figure 1.11 and discussed in Chapter 1.4.

Prior to loading the samples into the electrophoresis tray, 5 ± 0.1 μL of loading dye was added to each PCR tube to enable a better visualization of the sample being loaded and also to weigh down the DNA and keep it at the bottom of the well. The dye (purchased through Fermentas) comes as a 6 X loading dye solution in 1 mL and consists of 10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol, 60% glycerol, and 60 mM EDTA. A one to six dilution of the loading dye solution was prepared for the samples (1 part 6 X loading dye to 6 parts DI water). The dye also helps to determine how far the samples have traveled since the voltage was applied to the electrophoresis tray.

A GeneRuler™ 1 kb DNA ladder (purchased through Promega, G5711) was pipetted into the right-most well on the agarose gel. The DNA ladder acts as a ruler with known base-pair sizes to help estimate the base-pair sizes of the sample DNA fragments being separated.

Each well in the agarose gel was loaded with 13 ± 0.1 µL of PCR sample. The gel was then subjected to electrophoresis at 74 ± 1 V in a refrigerator at 4 ± 1 °C for 4 ± 0.1 hours. The electrophoresis run had its greatest resolution and separation between DNA segments under the conditions of 74 ± 1 V for 4 ± 0.1 hours at a temperature of 4 ± 1 °C. Figure 2.1 shows an idealized representation of the separation between DNA fragments after electrophoresis.

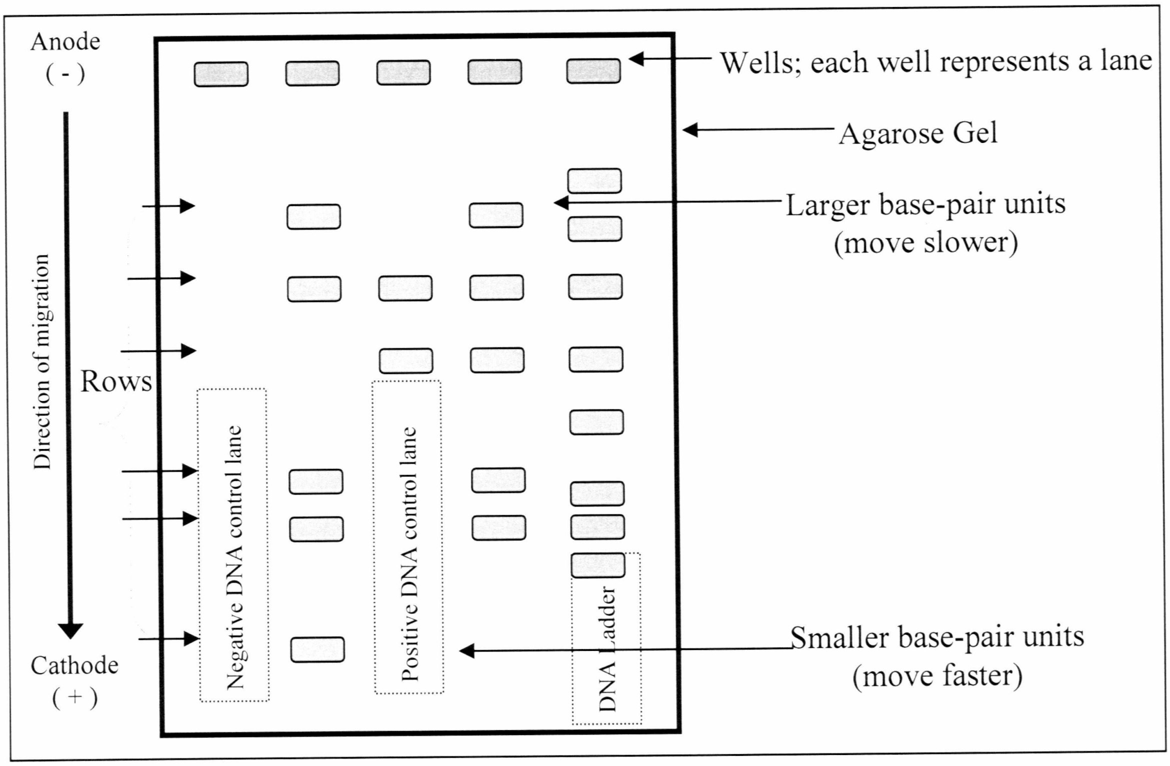

Figure 2.1. A representation of an ideal DNA separation on an electrophoresis gel shows a clear separation of the DNA fragment bands. The wells in an electrophoresis gel represent lanes. The direction of migration is from the anode to the cathode. The larger fragments migrate slower and therefore remain closer to the top of the gel while the smaller fragments are able to migrate through the agarose matrix faster. The lane where the negative DNA control was placed shows no bands while the lane where the positive DNA control was placed shows two bands towards the top of the gel. The size and migration speed of the positive control fragments depends on the type of positive control. Rows are compared to each other and the DNA ladder for base-pair size and to determine if a fragment of a given base-pair size appears at a given point.

2.6. Visualization

The electrophoresis gel containing the DNA segments was viewed and photographed under UV light using the Multi Image Light Cabinet ChemImager Ready and the Alpha Imager 2200 version 5.5 program. The ethidium bromide bound to the sugar-phosphate backbone of the DNA segments glows under UV light allowing the locations of the DNA segments on the gel to be determined. The resulting photographs of the visualized DNA are presented and interpreted in Chapter 3.

Chapter 3

RESULTS

The results of the UV visualization showed that DNA was recovered from each treated substrate sample (Figures 3.1 and 3.2). Therefore, fluorescein did not destroy the DNA in this study. This means that DNA can be recovered from latent blood after detection by fluorescein.

Figures 3.1 and 3.2 illustrate that DNA was extracted from the fluorescein-treated latent blood samples. The visible bands in lanes 2-6 and 8-12 of Figure 3.1 and lanes 3-4, 6, and 9-11 of Figure 3.2 illustrate that DNA fragments extracted from the samples are present on the gels. If DNA had not been extracted from the samples, the visualizations would have not shown fluorescent banding patterns on the gel except for the DNA ladder on the far right of the gel and the positive DNA control on the far left of the gel.

The positive (+) results in Table 2.1 represent the presence of DNA bands when the electrophoresis gels from the method (c) samples were viewed in the ultraviolet light cabinet. The negative (-) results correspond to no visible DNA bands on the gel. Methods (a) and (b) in Table 2.1 also produced positive DNA recoveries but, the results were visualized but not photographed.

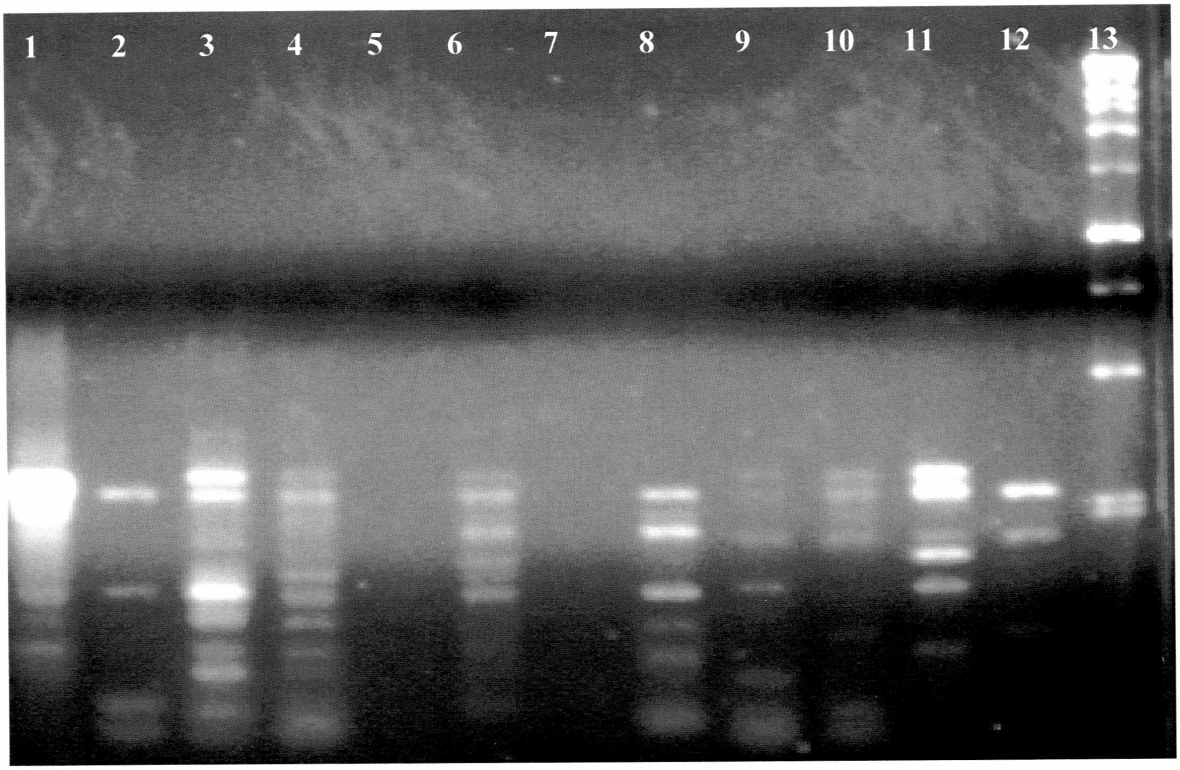

Figure 3.1. The results of a DNA separation after fluorescein use, chelex extraction, PCR amplification and gel electrophoresis appear under UV light. The lane representations are: (1) positive DNA control, (2) 1 part blood to 1000 parts DI water, (3) 1 part blood to 10 parts DI water, (4) 1 part blood to10 parts DI water, (5) negative DNA control, (6) 1 part blood to 10 parts DI water, (7) negative DNA control, (8) 1 part blood to 100 parts DI water, (9) 1 part blood to 100 parts DI water, (10) 1 part blood to 1 part DI water, (11) 1 part blood to 100 parts DI water, (12) 1 part blood to 1000 parts DI water, and (13) 1 kb DNA ladder. The visualized electrophoresis gel is the result of a DNA separation on a 2% agarose gel at 74 ± 1 V and approximately 4 ± 1 °C using method (c) in Table 2.1.

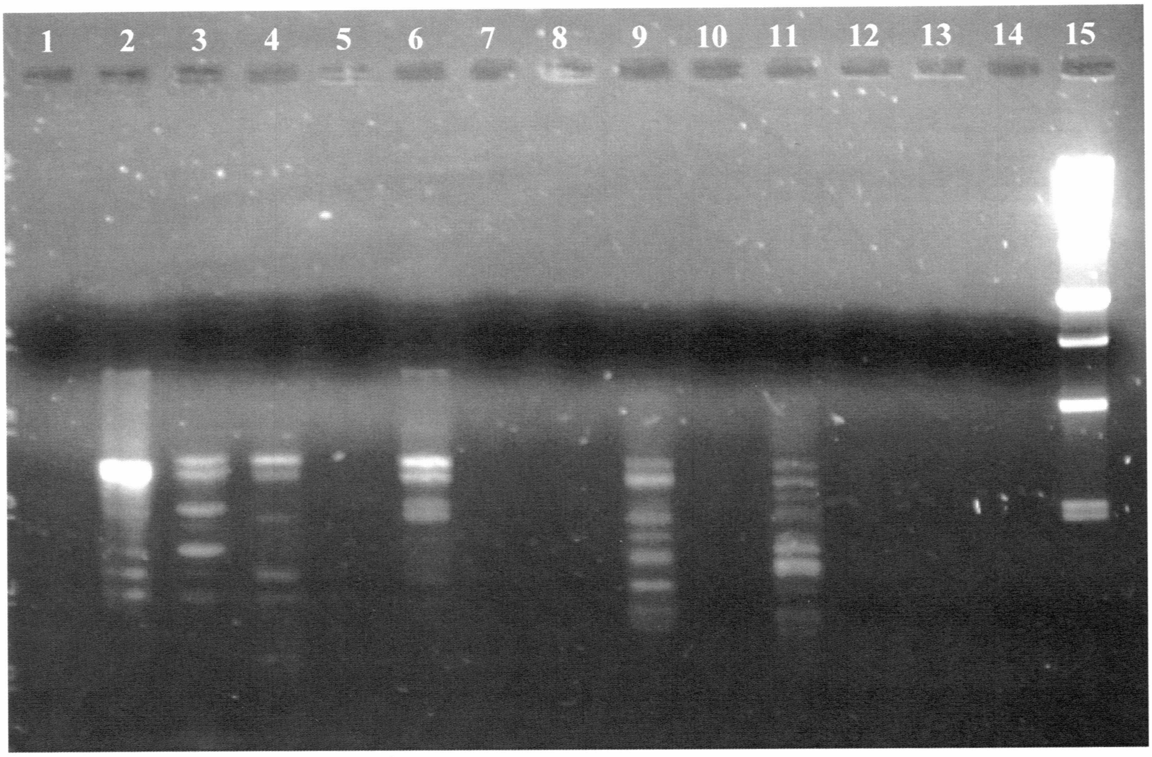

Figure 3.2. The results of a DNA separation after fluorescein use, chelex extraction, PCR amplification and gel electrophoresis appear under UV light. The lane representations are: (1) blank, (2) positive DNA control, (3) 1 part blood to 10 parts DI water, (4) 1 part blood to 1000 parts DI water, (5) negative DNA control, (6) 1 part blood to 100 parts DI water, (7) negative DNA control, (8) blank, (9) 1 part blood to 50,000 parts DI water, (10) blank, (11) 1 part blood to10,000 parts DI water, (12) blank, (13) blank, (14) blank, and (15) 1 kb DNA ladder. The visualized electrophoresis gel is the result of a DNA separation on a 2% agarose gel at 74 ± 1 V and approximately 4 ± 1 °C using method (c) in Table 2.1.

The blood to DI water ratios tested on the visualized gels ranged from 1 part blood to 1 part DI water to 1 part blood to 50,000 parts DI water. The fact that all of the samples had recoverable DNA indicates this method's sensitivity. The largest dilution tested in this study was 1 part blood to 50,000 parts water but it has been reported that fluorescein can detect blood at dilutions of up to 1 part blood to 100,000 parts water

[Budowle et al, 2000]. At any given violent crime scene, whole blood is deposited on various materials, so even when a clean-up of the crime scene is attempted, there is still generally more blood remaining at the scene than what was tested in this study [James, 2002].

The blood used for this study was from the same individual. Therefore, it was expected that all of the DNA bands would occur with the same intensities and banding patterns in each lane. However, this was not the case (see Figures 3.1 and 3.2). Even though each band represents a sequence of base pairs, some bands have greater intensities than others in the same row and different relative intensities to other bands in their lane when compared to the other lanes. This could be due to poor separation of the DNA fragments in which the bands bunch up and appear more intense. For example, the positive DNA control has a more intense banding and a larger spread than expected (see Figures 3.1 and 3.2). This is because the positive DNA control was in sufficient quantity to be observed by electrophoresis before amplification. Therefore, amplifying the positive DNA control caused too many DNA control segments to be placed on the gel. Poorly separated bands are more intense and thicker than the clearly-separated thin bands.

Figures 3.1 and 3.2 demonstrate that the techniques used to extract and visualize the DNA were not optimal. Ideally, the DNA bands would span the length of the entire gel (see Figure 2.1) yielding a better analysis of the separated products. Also, an inappropriate DNA ladder was used which prevented an accurate determination of base-pair size. Only the lightest band of the 1 kb DNA ladder, the 250, 253 bp band (shown in Figure 3.3), overlapped the sample fragments and could be used to estimate the base-pair sizes of a portion of the sample DNA.

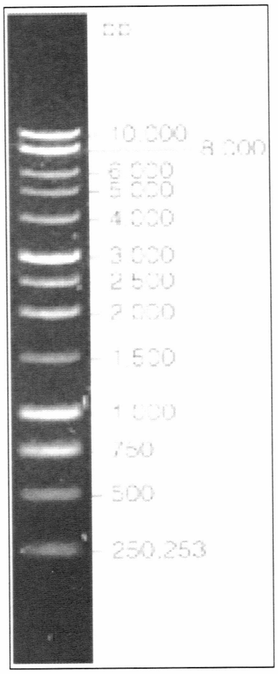

Figure 3.3. The 1 kb DNA ladder consists of 13 fragments with sizes ranging from 250 to 10,000 base pairs. The 1,000 and 3,000 bp fragments have increased intensity relative to the other bands on ethidium bromide-stained agarose gels and serve as reference indicators. All other fragments appear with equal intensity on the gel. [from Promega, 2005]

The base-pair sizes of two of the sample bands, corresponding alleles at 262 bp and 266 bp for the D18S51 locus, were able to be estimated using the 1 kb DNA ladder. However; the base pair sizes and corresponding alleles of the remaining bands of the samples could not be accurately identified. The DNA ladder that was used was what was available; future experiments will include a DNA ladder of the appropriate size. For locus D18S51, the base-pair range is between 262 bp and 342 bp, so a good DNA ladder would cover this expected base-pair range.

The base-pair sizes of a number of possible alleles (alleles are discussed in Chapter 1.2) are presented in Table 3.1. Table 3.1 also gives the 3' flanking sequence (where the primers flank the DNA template [as discussed in Chapter 1.3]) and the repeat structure (the base pair sequence [also discussed in Chapter 1.3]) associated with the D18S51 locus. This is important for understanding where the expected DNA bands should appear on the gel based on the reported allele size. The differences between some of those alleles are quite small in base-pair size, lending to the difficulty of separating and visualizing each allele.

Table 3.1. The PCR product sizes of observed alleles for the D18S51 locus were obtained from the FBI 13 CODIS Core STR Loci Fact Sheet [adapted from NIST, 2005].

Allele (Repeat #)	Size	3' Flanking Sequence
7	262 bp	
8	266 bp	AAAG AGAGAG
9	270 bp	AAAG AGAGAG
9.2	272 bp	
10	274 bp	AAAG AGAGAG
10.2	276 bp	
11	278 bp	AAAG AGAGAG
12	282 bp	AAAG AGAGAG
12.2	284 bp	
13	286 bp	AAAG AGAGAG
13.1	287 bp	
13.2	288 bp	AAAG AG AGAGAG
14	290 bp	AAAG AGAGAG
14.2	292 bp	AAAG AG AGAGAG
15	294 bp	AAAG AGAGAG
15.1	295 bp	
15.2	296 bp	AAAG AG AGAGAG
16	298 bp	AAAG AGAGAG
16.2	300 bp	
16.3	301 bp	
17	302 bp	AAAG AGAGAG

Table 3.1. (cont.)

Allele (Repeat #)	Size	3' Flanking Sequence
17.1	303 bp	
17.2	304 bp	AAAG AGAGAG
17.3	305 bp	
18	306 bp	AAAG AGAGAG
18.1	307 bp	
18.2	308 bp	
19	310 bp	AAAG AGAGAG
19.2	312 bp	AAAG AGAGAG
20	314 bp	AAAG AGAGAG
20.1	315 bp	
20.2	316 bp	
21	318 bp	AAAG AGAGAG
21.2	320 bp	
22	322 bp	AAAG AGAGAG
22.1	323 bp	
23	326 bp	AAAG AGAGAG
23.1	327 bp	
24	330 bp	AAAG AGAGAG
25	334 bp	AAAG AGAGAG
26	338 bp	AAAG AGAGAG
27	342 bp	AAAG AGAGAG

Some possible sources of the poor separation between the different alleles are that too high a voltage and/or too low percentage of agarose were used. A lower voltage might slow down the migration of the small DNA segments through the gel. A higher percent of agarose would also slow down the migration of these DNA fragments because the agarose matrix would become denser and it would be more difficult for the DNA fragments to pass through it. This would improve the separation between the smaller DNA fragments that were used in this study.

Another possible explanation of these results is that the annealing temperature could be too low. This can cause non-specific binding of nucleotides as discussed in Chapter 1.3. The annealing temperature used for this PCR amplification was 55 °C based on other studies [Budowle et al, 1995]. However, the annealing temperature for the primers used in this study was calculated to be 57 °C using the formula in Chapter 1.3. The calculated temperature should be used for annealing.

Each PCR reaction doubles the DNA target. As the PCR reaction continues, the product of the amplification accumulates, yet the supply of reagents becomes depleted and the amount of product produced per cycle declines. If there are too many PCR cycles, the amount of amplified product produced per cycle declines and becomes increasingly non-specific. [Saferstein, 2002]

Additional possible explanations for the observed non-idealities in the visualized gels include: 1) unequal sample quantities were loaded into the wells in the agarose gel, 2) the wells in the agarose gel were not of the same size, 3) impurities in the gel may be causing interferences and 4) PCR and electrophoresis are not 100% perfect [Duffy, 2005]. Further experiments are needed to test these hypotheses.

The results of this study demonstrate the recovery of DNA from latent blood after using fluorescein to identify the blood. The PCR method of amplifying the extracted DNA was useful yet not optimal. The electrophoresis separation of DNA segments was also useful yet not optimal. However, the purpose of this study was to determine if DNA could be recovered from latent blood identified with fluorescein, and it was achieved.

Chapter 4

CONCLUSIONS AND FUTURE WORK

This research found that fluorescein may be used at crime scenes to detect latent blood stains without destroying DNA evidence. This is an important advance in evidence collection and analysis that will enable suspect DNA to be matched to crime scene DNA found through the use of fluorescein.

The purpose of this study was to determine if DNA could be extracted from blood-stained denim samples after the use of fluorescein to identify the blood stains. The study's purpose was achieved and DNA was successfully extracted from the denim substrates after fluorescein use. The experimental methods used in this study involved applying blood to the denim substrates, drying the substrates, treating the substrates using fluorescein and hydrogen peroxide, extracting the DNA from the blood-stained denim using a chelex solution, amplifying the extracted DNA using PCR, separating the DNA by gel electrophoresis, and visualizing the DNA using UV light. The blood to DI water ratios tested ranged from 1 part blood to 1 part DI water to 1 part blood to 50,000 parts DI water. The fact that all of the samples had recoverable DNA indicates this method's effectiveness and sensitivity.

The next steps in this research should be to determine and optimize the sensitivity of the method and examine the effects of chemical interactions on the observed results. These steps will include optimizing the DNA amplification and separation, sequencing the DNA (see Chapter 1.5), identifying the chemical reaction mechanism by which fluorescein binds to the blood, and determining whether fluorescein will react with any other body fluids or compounds. For example, urine has been reported to react with fluorescein [Cheeseman, 1995] so methods for distinguishing the fluorescence of urine from that of blood need to be developed.

Optimizing the DNA PCR amplification procedure will require several modifications of the method used in this study. A prioritized list of these modifications follows.

First, every component used in the process, from the DNA template to the primers, must remain on ice until the PCR process has begun. The DNA samples, Master Mix, and primers were not kept on ice at all times. This is the most important improvement to the study's method because if the components are not kept on ice, it could lead to denaturing of the DNA or non-specific binding. Since the samples may be off ice for different amounts of time, they may be exposed to different temperature regimes and display different amounts of denaturing. This process is most likely the source of the non-uniform banding results.

Second, FailSafe™ PCR, an enzyme mixture designed to remove interferences, should be utilized. The FailSafe™ PCR contains a blend of thermostable DNA polymerases and a set of twelve reaction pre-mixes. The FailSafe™ PCR Enzyme Mix is an enzyme blend containing a 3' → 5' proofreading enzyme for high reliability. The pre-mixes contain a buffered salt solution with all four dNTPs, and various amounts of $MgCl_2$ and "FailSafe PCR Enhancer (with betaine)" [EPICENTRE Biotechnologies, 2003]. The steps involved in using FailSafe™ PCR include adding the DNA template, primers and the FailSafe™ PCR to the sample and subjecting the mix to PCR amplification. The presence of betaine (trimethyl glycine) in the FailSafe™ PCR Enhancer substantially improves the yield and specificity of amplification of many target sequences. In addition, betaine also may enhance PCR by protecting DNA polymerases from thermal denaturation [EPICENTRE Biotechnologies, 2003]. This is important to remove any impurities and inhibitors of the PCR reaction. There are many circumstances, such as non-sterile PCR tubes, that could have introduced impurities or inhibitors to the sample. This would result in less efficient PCR and extraneous bands.

Third, with each PCR reaction, the DNA target doubles. As the PCR reaction continues, the supply of reagents becomes depleted and the amount of product produced per cycle declines. If there are too many PCR cycles, the amount of amplified product produced per cycle declines and becomes increasingly non-specific and extraneous banding can appear [Saferstein, 2002]. It is important to decrease the number of PCR cycles so there are enough reagents available to complete the production of DNA copies. It is easier to decrease the number of cycles than it is to include larger amounts of reagents when working with small quantities of DNA; however increasing the amounts of reagents is another option. Another aspect would be to increase the extension time to make sure there is enough time to finish the extension.

Fourth, the annealing temperature should be decreased to 57 °C based on annealing temperature calculations. This increases the specificity of the binding and produces fewer extraneous bands (additional primers will require new annealing temperature calculations). Although the temperature programmed for annealing in this study was only 1 degree higher than the calculated temperature, it could have had an impact on how the primers annealed to the DNA template.

The last priority for amplification modifications is to check for temperature gradients in the thermocycler sample holder. The samples in this study were staggered throughout the sample holder, so if the thermocycler had a temperature gradient, it would have affected some of the samples ability to replicate by putting them under non-ideal temperatures. It is not known if the thermocycler in this study has a temperature gradient in its holder, but it is a potential cause of non-uniform banding.

PCR has many variables, so to confirm this optimization a follow-up comparative analysis should be done using the same procedures but manipulating one variable at a time.

Additionally, more than one locus should be used for analysis. A minimum of six loci should be analyzed, although analyzing thirteen loci is recommended. With additional loci, additional primers are needed. The primer sequences for all 13 CODIS loci can be taken from the FBI 13 CODIS core STR loci table [NIST, 2005]. All of the identified primers may be added to the DNA template using a multiplex system, which is a combination of primers prepared by a manufacturer. If preparing primers separately, longer sequences should be chosen (for example, greater than 20 base-pairs); this yields higher specificity of binding to the DNA template.

Optimizing the separation technique involves one major change; the percentage of agarose in the gel should be increased to an amount greater than 2%. With the base-pair sizes in this study, the amount of agarose was not enough to separate such small

DNA fragments across the gel. The DNA fragments did separate, but the separation occurred at the end of the gel. It is important to increase the amount of agarose to allow for small DNA fragment separation across the entire length of the gel.

Another potential way to improve the separation method is to determine if there is a temperature gradient across the gel during the electrophoresis process. If there is any temperature variation, this will cause the DNA fragments in the lanes to migrate at different speeds. However, this is not likely the case in this study as the DNA samples all migrated the same approximate distance.

A way to improve analysis of the results is to change DNA ladders. The 1 kb DNA ladder used in this study was too large to accurately identify any of the bands of the DNA samples. A DNA ladder that covers the range of expected base-pairs should be used. For locus D18S51, the expected base-pair range is between 262 bp and 342 bp, so a DNA that closely covers that range is important.

The visualized gel electrophoresis results for methods (a) and (b) in Table 2.1 were not photographed, so the results could not be quantified. Photographing the results will be important in future work for allowing a quantification of the analysis results. However, the results were similar to method (c), indicating that the application of fluorescein and hydrogen peroxide did not interfere with DNA recovery. These results also indicate that the extraction method used was effective.

To directly compare the effects of latent blood visualization techniques on DNA recovery, a parallel study of fluorescein and luminol should be conducted following the optimized methods described above. By subjecting samples to identical analytical techniques but different chemical treatments (luminol as compared to fluorescein), any observed differences will be due to the potentially different impacts of the chemicals.

This was a qualitative study; a quantitative study should follow. One method of conducting a quantitative study would be to quantify the amount of DNA prior to and after PCR using the Slot Blot test. The test uses an apparatus to immobilize DNA onto a nylon membrane. A probe, which recognizes human DNA, is hybridized to the membrane and produces a color or luminescence when exposed to human DNA. The product of the test is compared against a dilution series of DNA standards. [Saferstein, 2002] This would indicate the effectiveness of the PCR procedure. Another method for determining the effectiveness of the recovery procedure would be DNA sequencing. DNA sequencing is conducted after the PCR process and will show if the DNA has been degraded.

There are several other aspects of fluorescein that, if understood, would lead to a more effective use of fluorescein in the discovery of latent blood at crime scenes. An understanding of the chemical mechanism behind the reaction of fluorescein and blood could lead to the elimination of false positives and more effective methods for applying fluorescein to substrates. A study of what other substances cause fluorescein to fluoresce could also eliminate false positives. The kinetics of the reaction between fluorescein and blood, particularly the rate of decay of fluorescence will help investigators in planning for the appropriate photography and searching of the scene.

This technique, when properly characterized, will provide investigators with a valuable tool for the identification, collection, and analysis of latent blood evidence.

This tool will lead to the collection of more blood evidence that can be used in DNA recovery and sequencing and, hopefully, lead to more crimes being solved.

Chapter 5

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Glossary

9477A – Name of the positive human cell line control used in forensic DNA analysis [Rudin, 2002].

Agarose – Gel medium used for separation of DNA fragments in a variety of tests [Rudin, 2002].

Allele - Any of several alternative forms of a gene located at the same point on a particular pair of chromosomes. For example, the genes determining blood types A and B are alleles [Rudin, 2002].

Allele Frequency – The relative occurrence of a particular allele, or gene form, in a population [Rudin, 2002].

Amplification – The process of making multiple copies of a particular region of a DNA strand using PCR [The American Heritage® Dictionary of the English Language, 2000]

Angiography – An examination of blood vessels [The American Heritage® Dictionary of the English Language, 2000].

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Association – A concept used in crime scene reconstruction or a description of relationship between two objects, items, or people [Rudin, 2002].

Band – The visual image representing a particular DNA fragment [Rudin, 2002].

Band Size – The number of base pairs in a sequence of a DNA fragment [The American Heritage® Dictionary of the English Language, 2000].

Base Pair – Two complimentary bases held together by hydrogen bonds (A-T, C-G) [Rudin, 2002].

Biological Evidence - Any type of biological matter collected at a crime scene including: blood, urine, semen, feces, tissue, skin cells, decomposition fluid, saliva, tears, mucus, perspiration, vomit, and pus [James and Nordby, 2002].

Blood Spatter - The pattern made by the impact of spilled blood on a surface. The pattern of the impact can provide vital information about the source of the blood. Blood spatter can help determine the size and type of wounds, the direction and speed with which the perpetrator or victim was moving, and the type of weapons used to create the blood spill [James and Nordby, 2002].

Catalyst - A substance that accelerates the rates of chemical reactions but is not itself permanently changed by the reaction [The American Heritage® Dictionary of the English Language, 2000].

Centromere – The condensed region of each chromosome that appears during meiosis where the chromatids are held together to form an X shape [The American Heritage® Dictionary of the English Language, 2000].

Chromatid - One of the usually paired and parallel strands of a duplicated chromosome joined by a single centromere [The American Heritage® Dictionary of the English Language, 2000].

Chromosome - A rod-like structure in the nucleus of a cell along which genes are located made up of DNA [Rudin, 2002].

CODIS - CODIS stands for Combined DNA Index System. It is the Federal Bureau of Investigation (FBI) Laboratory's database that enables federal, state and local crime labs to exchange and compare DNA profiles electronically. This exchange makes it possible to link crimes to each other, and to convicted offenders. CODIS was made possible by the DNA Identification Act of 1994 that formalized the FBI's authority to create a national DNA index for law enforcement purposes. After CODIS identifies a potential match, qualified DNA analysts in the laboratories contact each other to validate the match [Rudin, 2002].

Degradation – The breakdown of DNA by chemical or physical means into smaller fragments [Rudin, 2002].

DNA - Deoxyribonucleic Acid. Constructed of a double helix, DNA is the genetic material contained in cells made up of nucleotides (adenine, guanine, thymine and cytosine). All organic matter has a specific DNA sequence; these sequences act like a genetic fingerprint and are currently the best method for identifying organic evidence like blood and other bodily fluids [The American Heritage® Dictionary of the English Language, 2000].

DNA Profiling - The process of DNA sequencing to identify DNA patterns or types. In forensic science this testing is used to indicate parentage or to exclude or include individuals as possible sources of bodily fluid stains (blood, saliva, semen) and other biological evidence (bones, hair, teeth.) [James and Nordby, 2002].

DNA Sequencing - The process of deciphering the precise order of nucleotide bases in a DNA molecule [James and Nordby, 2002].

Electrophoresis - A technique in which molecules are separated by their rate of movement in an electric field. In the case of DNA analysis, the fragments are separated by size [Rudin, 2002].

EDTA - Ethylenediaminetetraacetic acid. It is a crystalline acid that acts as a strong chelating agent and forms a sodium salt used as an anticoagulant and an antidote for metal poisoning [The American Heritage® Dictionary of the English Language, 2000].

Eukaryotic - A single-celled or multicellular organism whose cells contain a distinct membrane-bound nucleus [Rudin, 2002].

Evidence - Anything that has been used, left, removed, altered, or contaminated during the commission of a crime [James and Nordby, 2002].

False positive - A positive test result in a subject that does not possess the attribute for which the test is being conducted [The American Heritage® Dictionary of the English Language, 2000].

Fluorescence - The emission of electromagnetic radiation, especially of visible light, stimulated in a substance by the absorption of incident radiation and persisting only as long as the stimulating radiation is continued [The American Heritage® Dictionary of the English Language, 2000].

Forensic Science - The application of science to law and the investigation of criminal activity [James and Nordby, 2002].

Gene - A unit of inheritance located on a chromosome [The American Heritage® Dictionary of the English Language, 2000].

Genetic Marker – A defined location on a chromosome having known genetic characteristics [Rudin, 2002].

Genetic Profiling - The use of DNA technology to identify given individuals through their unique DNA sequences, also known as **genetic fingerprinting** [James and Nordby, 2002].

Globin Chain – A polypeptide chain [The American Heritage® Dictionary of the English Language, 2000].

Hemoglobin - A red blood cell protein responsible for transporting oxygen in the bloodstream. It also provides the red coloring of blood [The American Heritage® Dictionary of the English Language, 2000].

Intercalates – To insert [The American Heritage® Dictionary of the English Language, 2000].

Latent - Refers to something invisible to the naked eye, such as finger, palm or footprints [James and Nordby, 2002].

Latent Evidence – Evidence that is not visible to the naked eye such as fingerprints [James and Nordby, 2002].

Locus - (Loci, pl.) The specific physical location on a chromosome [Rudin, 2002].

Luminescence - The emission of light from a molecule or atom in an electronically excited state [The American Heritage® Dictionary of the English Language, 2000].

Luminol - A chemical that is used to identify latent blood and is capable of detecting bloodstains diluted up to 10,000 times [James and Nordby, 2002].

Match – When genetic profiles show the same types at all loci tested and no unexplainable differences exist [Rudin, 2002].

Meiosis - Cell division that produces reproductive cells in sexually reproducing organisms. In meiosis, the nucleus divides into four nuclei each containing half the chromosome number [The American Heritage® Dictionary of the English Language, 2000].

Molecular Weight – The molecular mass of a molecule. In DNA analysis, "molecular weight" and "band size" are used interchangeably [Rudin, 2002].

Molecular Weight Marker – DNA fragments of known size from which the size of an unknown DNA sample can be determined [Rudin, 2002].

Monochromatic Light - Light having a single wavelength or frequency [The American Heritage® Dictionary of the English Language, 2000].

Nucleic Acid - single or double-stranded chains of four different kinds of nucleotides joined one after the other at their phosphate groups [Weaver, 2002].

Physical Evidence - Any object that can establish that a crime has been committed, or can provide a link between a crime and its victim, or between a crime and its perpetrator [James and Nordby, 2002].

pKa - The pKa value of an organic compound arises from the ionization of either an acid or base. The pKa of an acid (or protonated base) is simply expressed as:

$$\mathbf{pKa = -\log (Ka)}$$

and that for [H^+] as;

$$pH = -\log([H^+]).$$

Which together in conjunction with equilibria expressions above result in the Henderson-Hasselbach equation:

$$pH - pKa = \log([X^-]/[HX]) = \log([B]/[HB^+]).$$

This equation indicates that at given pH value, the basic form predominates if pKa < pH and the acid form dominates if the pKa > pH.

Polymerase Chain Reaction (PCR) - A technique that allows small pieces of DNA to be cloned multiple times. The PCR process makes it possible to make millions of copies of a tiny original DNA sample so that there is more than enough DNA to test using common genetic marker analysis [The American Heritage® Dictionary of the English Language, 2000].

Porous – Full of or having pores [The American Heritage® Dictionary of the English Language, 2000].

Prophase - The initial stage of mitosis and of the mitotic division of meiosis characterized by the condensation of chromosomes consisting of two chromatids, disappearance of the nucleolus and nuclear membrane, and formation of the mitotic spindle [The American Heritage® Dictionary of the English Language, 2000].

Reagent - A substance taking part in a chemical reaction, especially one used to detect, measure, or prepare another substance [The American Heritage® Dictionary of the English Language, 2000].

Serology - Also known as "blood analysis" deals with the properties and actions of serums in blood. This was the preferred method of typing blood before the advent of DNA analyses [James and Nordby, 2002].

Serum - The liquid that separates from the blood when a clot is formed [Rudin, 2002].

Slot Blot – A diagnostic tool used in DNA analysis to determine how much DNA has been extracted from a sample. It is useful in making decisions about how much sample to use for various typing procedures [Rudin, 2002].

STR – Short Tandem Repeats, repeating units of an identical DNA sequence, where the repeat sequence unit is 2 to 5 bp in length. The repeat units are arranged in direct succession of each other, and the number of repeat units varies between individuals [Rudin, 2002].

Tandem Repeats – Repeating units of an identical (or similar) DNA nucleotide sequence arranged in direct succession in a particular region of a chromosome [Rudin, 2002].

Telomere – Either end of a chromosome [The American Heritage® Dictionary of the English Language, 2000].

Trace Evidence - Any type of physical evidence that may be collected from the crime scene, such as hair, fibers, soil, glue, paint, glass, or explosives residue. Trace evidence can sometimes be a source of DNA evidence. For example, an explosive device made from an empty plastic soft drink bottle may yield DNA evidence if the mouthpiece of the bomb remains intact [James and Nordby, 2002].

Transfer Theory – Whenever two human beings come into contact, something from one is exchanged to the other, ie dust, skin cells, hair etc known as Locard's Exchange Principle [James and Nordby, 2002].

Typing – Creating a genetic profile [James and Nordby, 2002].

Ultraviolet Light - Radiation, with wavelengths from 400 to 150 nm, which is invisible to the human eye and causes certain substances to shine brightly. Forensic scientists use ultraviolet light to reveal the presence of substances that are otherwise undetectable. [The American Heritage® Dictionary of the English Language, 2000]